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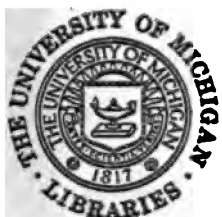
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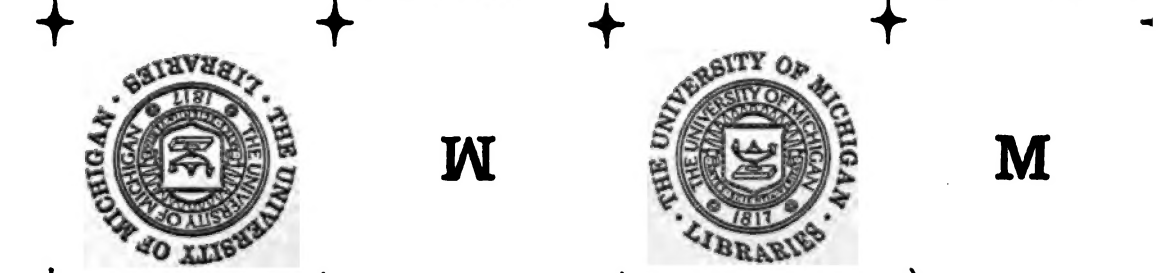
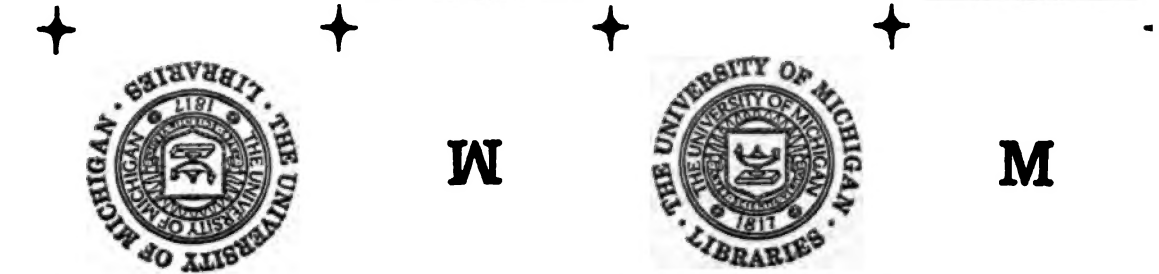
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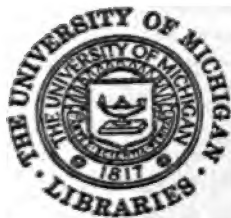
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Volume 153, Number 1, October 1976

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE

Volume 153, Number 1, October 1976

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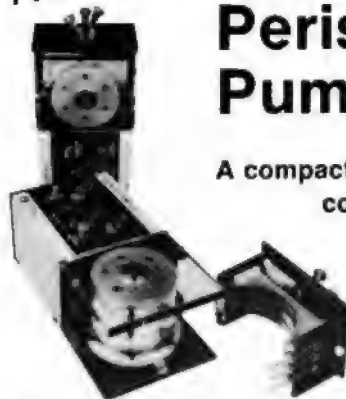
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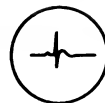
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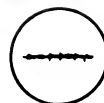
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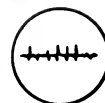
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ANNUAL REPORT

Annual Report of the Secretary-Assistant Treasurer and Managing Editor for the Year Ending December 31, 1975

Enclosed is the following is an abbreviated financial report prepared by Leo Kaden, C.P.A. of Padell, Kaden, and Co.

Balance of cash in banks at January 1, 1975	<u>\$ 35,126</u>	
Receipts:		
From "Proceedings":		
Subscriptions	\$132,999	
Advertising	9,932	
Publication charges	69,686	
Friends	2,975	
Profit sharing	3,391	
Royalty	<u>39</u>	
Total from "Proceedings"		219,022
Dues		47,417
Investment income, dividends and interest		20,192
Sundry receipts		82
Transfer in from investment accounts		<u>21,159</u>
Total receipts for the period		<u>307,872</u>
Total funds available		<u>342,998</u>
Disbursements:		
For "Proceedings":		
Academic Press	\$246,158	
Printing	<u>1,411</u>	
Total for "Proceedings"		247,569
Salaries and payroll taxes	42,836	
Office supplies, telephone, postage, insurance, etc.	6,519	
Meetings and travel	5,127	
Lectureships	1,053	
Pension (not funded)	2,400	
Investment advisory service	1,473	
Dues	<u>1,500</u>	
Total disbursements for the period		<u>308,477</u>
Balance of cash in banks at December 31, 1975		<u><u>\$ 34,521</u></u>

PROJECTED BUDGET CALENDAR YEAR 1976

Receipts:	
Subscriptions	\$133,500
Advertising	6,800
Publication charges	70,200
Friends	4,000
Total from "Proceedings"	<u>\$214,500</u>
Dues	34,100
Dividends and interest	<u>18,000</u>
Total receipts	<u>\$266,600</u>
Disbursements:	
Printing	\$ 2,500

ANNUAL REPORT

Academic Press	229,000
Salaries and payroll taxes	30,000
Office supplies and expenses	7,200
Meeting and travel	5,500
Accounting fees	700
Investment Advisory Services	1,500
Lectureships	2,400
Pension	2,400
Nat'l. Society for Med. Research	1,500
	<u>\$282,700</u>

Excess of expenditures over income \$ 16,100

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of Dr. D. W. Watson as President for 2 years beginning January 1, 1976; Dr. DeWitt Stetten, President-Elect; Dr. G. W. Siskind, Treasurer and Dr. M. R. Nocenti, Secretary-Assistant Treasurer for a similar period.

The following were elected members of the Council for a period of 4 years: Drs. D. L. Azarnoff, P. P. Foa, J. H. Leathem, R. J. Peanasky, M. D. Siperstein. Dr. N. B. Schwartz has been elected for a period of 2 years.

Tellers. Drs. M. Blank and R. Emmers.

The Society was represented by Dr. V. Glaviano at the meeting of the National Council of the National Society for Medical Research.

Miss Felice O'Grady has been in charge of the duties of the Society's National Office.

ACTIVE SECTIONS

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Dr. Bozidar Stavric
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Effects of Sodium Pentobarbital or Ether Anesthesia on Spontaneous and Electrochemically-Induced Gonadotropin Release¹ (39469)

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is well established in rats, that LH and rise concomitantly on the afternoon of strus (1-3) and that injection of sodium barbitol (Nembutal) shortly before the of the spontaneous gonadotropin prevents this release from occurring (butal-blockade) (4-7). Although electrochemical stimulation (ECS) (8) of the al preoptic area (MPOA) or arcuate-an eminence region (ARC-ME) is effective in inducing the release of gonadotropin in Nembutal-blocked proestrous rats, temporal patterns of LH and FSH secretion do not mimic those which occur spontaneously (6, 9). In most studies, following of the MPOA, plasma LH levels have decreased and are approaching basal concentrations prior to the commencement of a rise in FSH. Conversely, administration of hypothalamic extracts induced an immediate elevation of both gonadotropins *in vivo* (10) and *in vitro* (11). The following studies were undertaken to determine if the oral dissociation of LH versus FSH release following central nervous ECS was a result of the depressant effects of Nembutal on preoptico-hypothalamic function or was due to other factors.

Material and methods. The adult female Sprague-Dawley rats (200-250 g) used in this study were purchased (A/R Madison, Md.) and maintained in a light and temperature (22-24°C) controlled room for 2-3 weeks prior to use. Lights were on from 0600-1800. Daily vaginal lavages were performed and only those rats which exhibited at least two consecutive 4-day estrous cycles were selected for study. On the day of vaginal proestrus, animals were divided into two groups:

Group A. spontaneous proestrous LH and

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FSH release. At 1100 on proestrus, 10 cyclic rats were anesthetized with ether and the right external jugular vein was exposed. A polyethylene cannula (o.d. 0.038 in.) was inserted to the level of the right atrium. The cannula was coursed beneath the skin to the back of the neck, leaving 2-3 inches exposed for subsequent blood collections. Beginning at 1300, hourly blood collections (0.5 cc) were made for 6 h.

Group B. electrochemical stimulation of either the dorsal anterior hypothalamic area (DAHA) or the medial preoptic area (MPOA) at 1300 employing Nembutal anesthesia. Fifteen minutes before the anticipated spontaneous surge of LH/FSH, which occurs in our rat colony between 13-1500, an ovulation-blocking dose of Nembutal (30 mg/kg ip) was administered to a group of 31 animals. The right external jugular vein was cannulated as described in Group A. The animals were immediately placed in a stereotaxic apparatus and a blood sample (0.5 cc) was collected. A concentric bipolar stainless steel electrode [specifications previously described (4)] was stereotaxically oriented into either the right DAHA ($n = 23$) or the right MPOA ($n = 8$). A current intensity of 60 μ A/60 sec was supplied by a constant current stimulator (Electronics for Life Sciences, Rockville, Md.) and monitored by a cathode ray oscilloscope. Following stimulation, blood samples (0.5 cc) were collected at 60, 120, 180, 240, 300 and 360 min. An equal volume of 0.9% saline was reinfused after each blood collection. The following morning, the animals were sacrificed and the Fallopian tubes were examined for the presence of ova. Brains were perfused via the common carotid artery, first with 20 ml of 0.9% saline, saturated with potassium ferro-ferricyanide, and then with 20 ml of 10% formalin. Brains were removed, stored in 10% formalin.

lin and eventually were frozen-sectioned with a sliding microtome at a thickness of 60 μ to determine the stimulus size and diameter. Lesion diameters were measured with an ocular micrometer from the lateral extent of the area of electrocoagulation produced by ECS.

Group C. electrochemical stimulation (ECS) of either the MPOA or the DAHA 120 min after Nembutal injection. As in Group B, Nembutal (30 mg/kg ip) was administered to a group of 14 proestrous rats at 1245. The right external jugular vein of these animals was cannulated and a blood sample (0.5 cc) was collected at 1300 (0 time). Thereafter, the rats remained undisturbed (except for a blood collection at 60 min) until 1500 (120 min) at which time the MPOA of seven animals was unilaterally electrochemically stimulated (60 μ A/60 sec). The DAHA of the remaining seven rats was electrochemically stimulated (60 μ A/60 sec) and all experimental procedures described in Group B were then performed in both subgroups.

Group D. ECS of either the MPOA or the DAHA in ether-anesthetized proestrous rats. At 1100, 2 hrs before the anticipated onset of the spontaneous surge of gonadotropins, 11 rats were anesthetized with ether and the right external jugular vein was cannulated. Animals were immediately placed in a stereotaxic apparatus and a blood collection (0.5 cc) was taken (0 time). In each preparation, either the right MPOA ($n = 6$) or the right DAHA ($n = 5$) was electrochemically stimulated. Following ECS, blood samples were collected at 30, 60, 90, and 120 min. The following morning (estrus), animals were sacrificed and their Fallopian tubes were examined for the presence of ova; the brains were perfused, removed, and then were stored for future determination of stimulus size and location.

Radioimmunoassay procedures. Plasma LH was determined by radioimmunoassay according to the ovine:ovine procedure of Niswender *et al.* (10). A crude rat pituitary extract prepared to correspond with a B160 standard supplied to us by Dr. V. L. Gay was used as standard reference material; this preparation has been shown to have a LH potency of $0.17 \times$ NIH-LH-S1. Plasma

FSH was assayed by the rat radioimmunoassay kit materials generously provided by the NIAMDD and values are expressed in terms of the NIAMDD Rat-RP-1 which is $2.1 \times$ NIH-FSH-S1 standard. The kit materials were employed using modifications previously described (4) to permit the simultaneous assay of LH and FSH in small volumes of plasma. Statistical evaluations between and among groups was performed using Duncan's multiple range test (11).

Results. Group A. Spontaneous release of FSH and LH on proestrus. At 1400 both LH/FSH were significantly elevated above the basal plasma concentrations obtained at 1300 proestrus (Fig. 1A). One hour later, a further significant increase in both gonadotropins was observed and at 1600 LH/FSH had reached peak concentrations. Thereafter, LH decreased to basal concentrations by 1900 while plasma FSH remained elevated. At autopsy the following morning, all animals had ovulated and the Fallopian tubes contained 8–13 ova.

Following the injection of Nembutal at 1245, these spontaneous surges were blocked and LH/FSH concentrations remained at basal levels throughout the afternoon of proestrus. These plasma patterns and concentrations of LH/FSH have been reported previously (4).

Group B. ECS of the DAHA or the MPOA of Nembutal-anesthetized rats. The first significant ($P < 0.05$) increase in plasma FSH in DAHA-ECS rats did not occur until 120 min poststimulation and peak plasma FSH concentrations (274 ± 11 ng/ml) were observed by 180 min poststimulation. Once such peak concentrations were achieved, plasma FSH remained elevated in this and all other experimental groups throughout the remainder of the collection periods.

In DAHA-ECS rats, plasma LH concentrations remained at basal levels except for a slight but significant ($P < 0.05$) rise 120 min poststimulation. These animals had not ovulated by the following morning (estrus).

Following MPOA stimulation of a second group of rats, plasma LH began to rise at 30 min and peak plasma LH concentrations were obtained at 60–120 min poststimulation (Fig. 1B). These levels had de-

ed to baseline by 180–240 min. In con-, the first significant ($P < 0.05$) eleva- in plasma FSH in these animals was achieved until 120 min and peak concentrations were not reached until 180 min stimulation. The Fallopian tubes of DA-electrochemically stimulated rats gained 8–12 ova at autopsy the follow- morning. The locations of MPOA and DA stimulation sites are illustrated in 3.

Group C. ECS of the MPOA or DAHA minutes after Nembutal injection.

Unilaterally electrochemically stimu- lated in the DAHA 120 min after Nembu- tal administration exhibited significant ($P < 0.05$) elevations in plasma FSH concen- trations by 60 min and peak plasma levels (321 ± 14 ng/ml) by 120 min poststimula-

Plasma LH concentrations in these animals remained unaltered throughout entire blood collection periods. None of the animals had ovulated at autopsy on the following morning.

Unilateral ECS of the MPOA 120 min following the injection of Nembutal induced release patterns of FSH similar to those observed after DAHA-ECS. Signifi-

($P < 0.05$) plasma elevations of this gonadotropin were observed by 60 min and peak concentrations occurred (321 ± 56 ng/

ml) by 120 min poststimulation (Fig. 2A). Peak FSH plasma concentrations in this MPOA-electrochemically stimulated group were significantly greater ($P < 0.05$) than those observed in the DAHA group stimulated 120 min after Nembutal treatment. The maximal plasma LH concentrations observed 60 min poststimulation (124 ± 26 ng/ml) had declined to basal levels by 180–240 min poststimulation. Examination of the oviducts the following morning revealed that full ovulations had occurred (8–12 ova).

Group D. ECS of the MPOA or DAHA in ether-anesthetized proestrous rats. Unilateral ECS of the DAHA in ether-anesthetized rats at 1100 induced a significant ($P < 0.05$) release of pituitary FSH by 60 min and maximal plasma concentrations (302 ± 40 ng/ml) were achieved by 120 min poststimulation. Not only had plasma LH levels risen significantly from baseline by 60 min but they were further elevated by 120 min poststimulation. All animals in this group had ovulated completely by the following morning.

Unilateral ECS of the MPOA in ether-anesthetized rats elicited a significant ($P < 0.05$) release of both gonadotropins by 60 min poststimulation (Fig. 2B). Plasma LH and FSH concentrations increased simul-

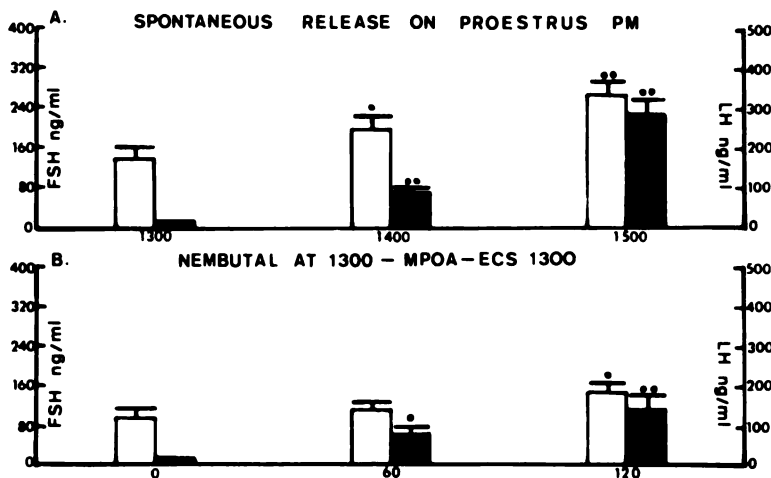


Fig. 1. Temporal and quantitative changes in plasma LH (shaded bars) and FSH (open bars) on the afternoon of proestrus at 13, 14, and 1500 (A), or 0, 60, and 120 min following electrochemical stimulation of the medial preoptic area (MPOA) at 1300 in Nembutal-treated proestrous rats (B). Vertical bars represent the standard error of the mean. (. = $P < 0.05$; .. = $P < 0.01$)

ANESTHETICS AND GONADOTROPIN RELEASE

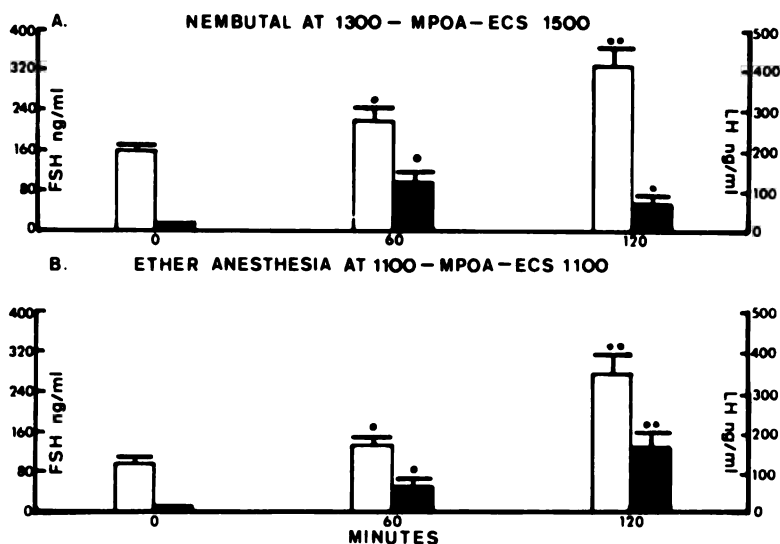


FIG. 2. Plasma concentrations of LH (shaded bars) and FSH (open bars) at 0, 60, and 120 min following electrochemical stimulation of the medial preoptic area (MPOA-ECS) in Nembutal-treated proestrous rats (A) or 0, 60, and 120 min after MPOA-ECS in etherized proestrous rats at 1100 (B). Vertical bars represent the standard error of the mean. (* = $P < 0.05$; ** = $P < 0.01$).

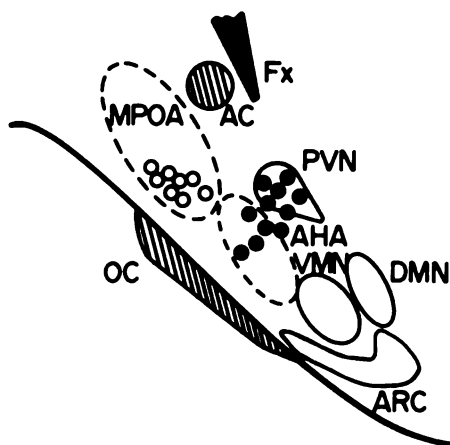


FIG. 3. Parasagittal section of the MPOA-hypothalamus of rats indicating centers of lesions produced following electrochemical stimulation (ECS) of the medial preoptic area (MPOA) (open circles) or the dorsal anterior hypothalamic area (DAHA) (black circles).

taneously thereafter. All animals had ovulated 8-13 ova by estrous morning.

Discussion. Electrochemical (9, 14) or electrical stimulation (5) of the MPOA elevates plasma LH concentrations within 30 min poststimulation and the present studies confirm these earlier observations. Since hypothalamic extracts injected directly into the pituitary portal vessels (10) pro-

voke an increase in plasma LH within 10 min, the apparent delay in the LH plasma rise in MPOA-electrochemically stimulated animals must occur somewhere within the preoptico-hypothalamic system. Examination of the temporal patterns of plasma LH/FSH which occur spontaneously on the afternoon of proestrus has revealed that both gonadotropins rise concomitantly (1-3). In contrast, electrochemical stimulation of the MPOA, the arcuate-median eminence (ARC-ME), or the DAHA in Nembutal-anesthetized rats induces an increase plasma FSH but the lag time from stimulation to the first significantly detectable plasma elevation in this gonadotropin was 1.5-2 hr. (4, 6, 9). Since Nembutal does not alter the responsiveness of the pituitary gland to LH-RH injections (15), it must have a central site of action in affecting gonadotropin release. If the interval from injection of Nembutal to ECS of the MPOA or the DAHA is delayed for 120 min, the time to the first rise in plasma FSH is markedly reduced. At this time, the animals are recovering from the anesthetic properties of Nembutal for they will exhibit withdrawal reflexes to foot-pinch, exhibit corneal reflexes, and show other general indications of behavioral arousal. Electrochemical stimulation

the CNS during this period effectively causes a more rapid release of pituitary LH than that which occurs in the deeply anesthetized preparation.

Consequently, it seems apparent that the neural regions which regulate FSH are highly and selectively sensitive to the depressant actions of Nembutal. Since

drug actions occur regardless of whether the MPOA or the DAHA is electrochemically stimulated, and the stimulus-release of pituitary LH is not appreciably altered by Nembutal, it is probable that this barbiturate affects MPOA or MPOA synaptic transmission at the level of the ARC-ME. Neuropharmacological studies have shown that central neural pathways which have abundant interneuronal connections such as the ascending reticular activating system are particularly vulnerable to the depressant actions of barbiturates (16).

It has been reported previously that the effect of ether anesthesia prior to the onset of the spontaneous surge of LH does not interfere with the release of this gonadotropin in the afternoon of proestrus (17). In the present study, ECS of DAHA at 1100 in ovariectomized proestrous rats elicits a release of LH within 60 min post-stimulation as compared to the 90–120 min delay observed in Nembutal-anesthetized preparation.

The results of these studies require a further evaluation of whether one or several releasing hormone(s) for LH/FSH exist since temporal plasma patterns of LH are unaltered by Nembutal in MPOA-electrochemically stimulated rats, and pituitary LH/FSH are released concomitantly following an i.v. injection of hypothalamic extracts, the present data support the concept that separate releasing hormones exist for these gonadotropins. Although the primary discharge of FSH eventually occurs in MPOA- or DAHA-electrochemically stimulated rats after induction of surgical anesthesia, it should be recalled that ECS produces an irritative lesion of the pituitary which continues to stimulate even in the absence of current flow. Thus, as recovery from anesthesia proceeds, this stimulative lesion could activate those neural components responsible for the release of FSH and this event takes

from 90–120 min poststimulation to occur.

Summary. These studies have examined the effects of sodium pentobarbital or ether anesthesia on temporal plasma patterns and concentrations of FSH and LH following electrochemical stimulation (ECS) of the medial preoptic area (MPOA) or dorsal anterior hypothalamic area (DAHA) in proestrous rats. The injection of sodium pentobarbital at 1245 on proestrus and ECS of either the MPOA or DAHA at 1300 elicited a rise in plasma FSH 120 min poststimulation. Injection of this barbiturate at 1245 and ECS of either region at 1500, when the effects of anesthesia had begun to wane, reduced the time interval between stimulation and the first significant plasma FSH rise to 60 min. A third group of proestrous rats was anesthetized with ether at 1100 and the DAHA or the MPOA was electrochemically stimulated. In such preparations, FSH in plasma rose significantly 60 min post-stimulation. In all MPOA-electrochemically stimulated groups, the first significant rise in plasma LH occurred 30 min poststimulation regardless of the time of treatment or anesthetic drug employed. In none of the groups did DAHA-ECS provoke a rise in plasma LH. These results suggest that the CNS systems controlling FSH but not LH secretion are sensitive to sodium pentobarbital and that the lag time between ECS and plasma rises in FSH apparently is related to the depressant effects of the barbiturate on neural FSH controlling systems.

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Vasopressor Potentiator for Norepinephrine in Hypertensive Rats (39470)

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Recently, attention has been given to a responsiveness to vasopressor agents in various forms of hypertension. Hyperand/or "water-logging" of blood vessels associated with prolonged hypertension has been found to narrow vessel lumen and lead to such hyperresponsiveness (1). In addition, subnormal monoamine oxidase activity accompanied by increased tissue norepinephrine concentrations has been reported in genetically hypertensive turkeys, which also exhibited a vasopressor response to exogenously administered norepinephrine (2).

Recently there has been a renewed interest in a "sensitizing factor" to catecholamines in hypertension, but because of the nature of some studies and the limited utilization of inadequate controls, reports have been conflicting. Several investigators have found evidence for a vasopressor agent potentiating factor in hypertensive subjects (3-6), and a circulating humoral factor has been suggested by a study that utilized the paraventricular union of a strain of rats that was highly susceptible to the development of hypertension (7). In addition, a blood factor in hypertensive humans that mimicked normal assay animals to the vasopressor effects of norepinephrine and angiotensin II has been reported (8). The present study was conducted to determine whether a factor exists in the serum of salt-induced hypertensive rats that would enhance the vasopressor activity of exogenously administered norepinephrine in sensitive bioassay animals.

Materials and methods. Development of experimental hypertension. Sixty-eight rats, initially weighing 166 ± 29 g, were quartered in a climate controlled $25^\circ \pm 2^\circ$ and 50-60% relative humidity 12 hr of light each day (6 AM to 6 PM). Animals were divided into three

groups of 20-24 animals and placed on diets consisting of Purina Lab Chow with NaCl added to make up 1.3, 5.6, and 8.4% of the ration by weight.¹ Deionized water and feed were provided *ad libitum*. The animals' weights and systolic blood pressures were recorded weekly with a programmed electrospphygmomanometer² and a cuff over the caudal artery. At the end of 225 days on the diets, the animals were sacrificed by decapitation, and their trunk blood was collected and allowed to clot at room temperature. Cells were separated from the serum by centrifugation, and the serum was stored at -28° until used. Serum samples were selected from the three dietary groups in such a manner that animals with a wide range of blood pressures were represented in the data.

Bioassay for norepinephrine response. A separate group of male rats weighing 300-400 g, which had previously been screened for blood pressure abnormalities, was used for the bioassay. After bilateral nephrectomy and a 24 hr recovery period, these animals were given dial-urethan³ as an anesthetic agent (1.8 ml/kg, ip), and pentolinium tartrate⁴ was given as an adrenergic blocking agent (5-8 doses of 0.25 mg/25 μ l each given iv at 5 min intervals). During the assay, blood pressures were measured via a polyethylene cannula (PE-50)⁵ placed in the internal carotid artery connected to a P-1000A pressure transducer and recorded using a Physiograph DMP-4A² recorder. A Narco SW-4 Servo-

¹ Nutritional Biochemicals Corp., Cleveland, Ohio.

² Narco Biosystems, Houston, Texas.

³ Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

⁴ Wyeth Laboratories, Philadelphia, Pennsylvania.

⁵ Intramedic tubing, Beckton-Dickinson, Rutherford, New Jersey.

Writer² accessory was used to expand the mean blood pressure recording scale for greater convenience in reading pressure changes. Intravenous injections were made through PE-20 cannulas³ inserted into both internal jugular veins. The cannula used for injecting norepinephrine was maneuvered into the superior vena cava. Systolic and diastolic pressures, electrocardiogram, and respiration were observed periodically throughout the procedure.

After the administration of the adrenergic blocking agent, a norepinephrine dose response curve was recorded for each animal and used to determine the quantity of norepinephrine required to produce a 10 mm Hg increase in mean blood pressure. The amount thus determined for each animal was subsequently used as a standard dose to be injected iv at 6 min intervals for the duration of the assay. Very small norepinephrine injection volumes were used to avoid the expansion of the extracellular fluid volume that would otherwise accompany multiple injections in the same animal. The mean volume for each injection of norepinephrine was $0.77 \pm 0.07 \mu\text{l}$ (SE) with a total mean volume injected into assay animals of 13.05 ± 1.45 (SE) μl . Responses to the standard dose of norepinephrine were considered acceptable if six consecutive responses varied no more than ± 1 mm Hg mean blood pressure, recorded from a steady blood pressure baseline, varying no more than ± 1.5 mm Hg. After the standard response was established, 15–25 μl of the serum sample was injected through a jugular vein cannula, and the standard dose of norepinephrine was repeated until it became apparent that no further increase in vasopressor response would occur. The three highest consecutive responses to the standard norepinephrine dose, recorded from a mean blood pressure baseline varying no more than ± 3 mm Hg, were selected and averaged, and their mean was used as the test response value.

Statistical analysis of data. Significance of the results obtained was determined by analysis of variance, Fisher's "F" test (9).

Results. Effect of diet upon blood pressure. Blood pressure ranges of the animals after 225 days on the 1.3, 5.6, and 8.4% NaCl diets are shown in Fig. 1. The mean

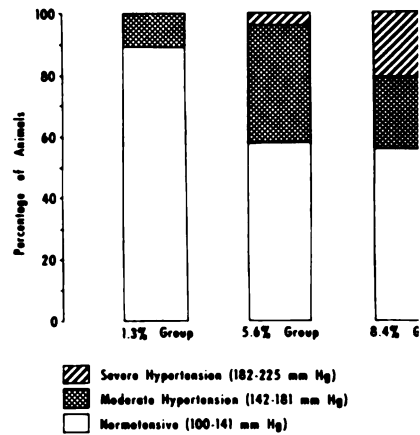


FIG. 1. Systolic blood pressure ranges of 1.3, 5.6 and 8.4% NaCl diets. The proportion of animals on the 5.6 and 8.4% diets that developed hypertension was similar. The severity of hypertension increased as dietary NaCl increased.

systolic blood pressures for the three groups were 129 ± 5 mm Hg (SE), 146 ± 5 mm Hg, and 154 ± 2 mm Hg, respectively. Eighty-nine percent of the animals on the 1.3% NaCl diet remained normotensive while only 46–48% of the animals on the high salt diets remained normotensive. Greater dietary salt content had little effect upon the percentage of animals developing hypertension (systolic BP > 142 mm Hg) but several of the animals developed severe hypertension (systolic BP > 182 mm Hg) on the 8.4% NaCl diet.

Effect of serum injection upon mean blood pressure. Ganglionic blockade reduced basal vascular tone with a resultant reduction in mean blood pressure to near 50 mm Hg. An hour or more was required for equilibration following the blocking agent, after which the blood pressure baseline remained stable for several hours. The administration of hypertensive or normotensive sera did not affect basal mean blood pressure; this may be seen in Table I.

Effect of serum injection upon norepinephrine response. The injection of serum from hypertensive rats resulted in an augmented response to standard doses of norepinephrine. After the administration of hypertensive sera, the vasopressor response to norepinephrine ranged from a 7% decrease to a 78% increase in response with a

I. BLOOD PRESSURE CHANGES OCCURRING DURING BIOASSAY PROCEDURE INDUCED BY GANGLIONIC BLOCKING AGENT, TEST SERUM, AND NOREPINEPHRINE. NOREPINEPHRINE VASOPRESSOR RESPONSE TO HYPERTENSIVE SERUM WAS HIGHLY SIGNIFICANT ($P < 0.001$).

Bioassay Rat							
Serum donor systolic BP in mm Hg	Body Weight in g	Systolic BP in mm Hg	Mean blood pressure		BP response to norepinephrine in mm Hg		Norepinephrine response change (%)
			Shortly after blocking agent in mm Hg	After serum in mm Hg	Control	Serum test	
120	370	—	68	44	11.0	11.0	0
117	350	—	78	50	17.2	16.8	-2
140	345	90	43	40	16.2	17.7	9
120	345	90	43	40	17.2	16.5	-4
117	350	115	53	53	15.8	14.8	-6
125	440	120	40	41	9.0	11.8	31
127	370	100	48	48	12.8	14.7	15
127	370	100	48	48	13.3	20.0	50
120	335	120	40	44	14.3	14.6	2
132	335	120	40	44	14.8	14.7	-1
)							
125 ± 7	361 ± 31	107 ± 13	50 ± 13	45 ± 4	14.2 ± 2.7	15.26 ± 2.66	9.4 ± 18%
)							
225	300	110	53	53	10.0	13.3	33
225	360	86	57	58	8.8	12.2	39
190	410	125	43	43	8.3	13.2	59
190	385	—	58	47	8.7	15.5	78
170	370	—	68	44	11.0	13.2	20
170	350	—	78	50	16.8	22.5	34
160	560	—	80	44	8.2	10.2	24
170	350	115	53	53	15.3	22.2	45
225	440	120	40	41	9.2	13.0	42
218	500	118	53	51	9.7	11.3	17
218	430	110	67	67	15.3	18.8	23
170	385	100	44	42	8.3	8.3	0
170	385	100	44	42	8.2	8.8	8
142	360	100	60	42	10.3	13.7	32
170	310	105	50	62	12.2	19.0	56
170	335	120	40	44	9.6	9.0	-7
160	340	125	67	60	11.7	13.2	13
217	340	85	50	60	13.2	15.5	13
170	380	92	60	61	14.5	18.7	29
)							
186 ± 27	384 ± 64	107 ± 13	56 ± 12	51 ± 8	11.0 ± 2.8	14.3 ± 4.3	29.4 ± 21%

of $29.4 \pm 5\%$ (SE). When compared with the preserum injection response, no difference was found to be highly significant ($P < 0.001$) (see Tables I and II). The initial response to norepinephrine both before and after hypertensive sera administration is shown in Fig. 2. The injection of normotensive serum usually results in rapid augmentation of response which continued for 90–180 min and subsequently declined to the preserum value during the ensuing 90–180 min.

The smaller and less significant differences in vasopressor response were observed after injection of normotensive sera (see Tables I and II). An example of the norepinephrine response after the injection of normotensive serum is shown in Fig. 3. Responses for this group ranged

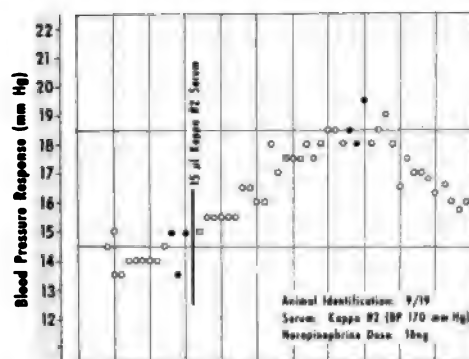
from a 5% decrease to a 50% increase with a mean increase of $9.4 \pm 6\%$ (SE), but the difference was not found to be significant ($P > 0.1$). Half of the normotensive group had a slightly negative or no increase in reactivity, as shown in Fig. 4, while only 11% of the hypertensive group had no augmentation.

Correlation of systolic blood pressure with norepinephrine response. Regression analysis of systolic blood pressures of serum donor animals and the subsequent vasopressor responses to the standard dose of norepinephrine found in bioassay animals revealed no significant correlation between the two ($P > 0.1$).

Discussion. Observations of a hyperresponsiveness to vasopressor agents occurring concomitantly with hypertension

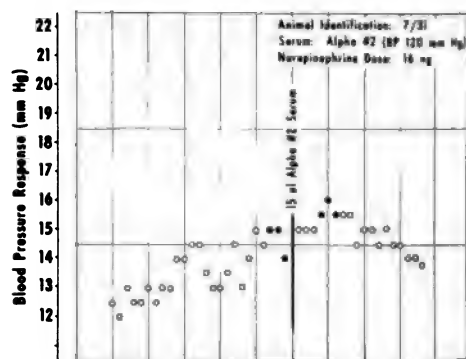
TABLE II. STATISTICAL *F*-TEST SUMMARY OF THE VASOPRESSOR RESPONSE TO NORMOTENSIVE AND HYPERTENSIVE SERA. THE CHANGE IN VASOPRESSOR RESPONSE ATTRIBUTED TO NORMOTENSIVE SERUM WAS NOT STATISTICALLY SIGNIFICANT WHILE THE RESPONSE ATTRIBUTED TO HYPERTENSIVE SERUM WAS HIGHLY SIGNIFICANT.

Normotensive serum	Degrees of freedom	Sum of squares	Mean squares	<i>F</i>	<i>P</i>
Between treatment sum of squares	1	27	27	$\frac{27}{8.7} = 3.1$	>0.1
Between animals sum of squares	7	292	41.7	$\frac{41.7}{8.7} = 4.8$	<0.05
Interaction sum of squares	7	61	8.7	$\frac{8.7}{.31} = 28$	<0.001
Between responses sum of squares	32	10	.31		
Total	47				
Hypertensive serum					
Between treatment sum of squares	1	310	310	$\frac{310}{6.2} = 50$	<0.001
Between animals sum of squares	18	1296	72	$\frac{72}{6.2} = 11.6$	<0.001
Interaction sum of squares	18	113	6.2	$\frac{6.2}{.39} = 15.9$	<0.001
Between responses sum of squares	36	14	.39		
Total	73				



Norepinephrine Injections At Six Minute Intervals

FIG. 2. Norepinephrine response after injection of hypertensive serum. Each circle represents a 16 ng dose of norepinephrine injected at 6 min intervals. The mean of the last three responses before injection of serum (solid circles) was used as control value while the mean of the three highest consecutive responses after serum (solid circles) was used as the test value. In this animal there was a 29% increase in response.

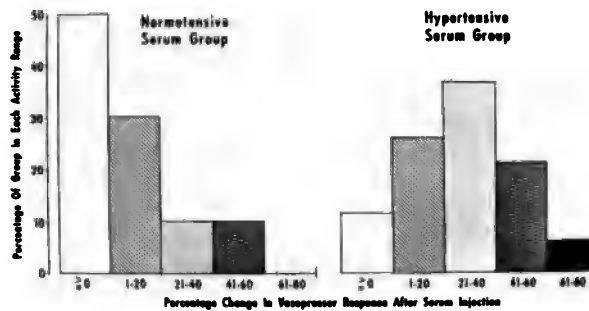


Norepinephrine Injections At Six Minute Intervals

FIG. 3. Norepinephrine response after injection of normotensive serum. Each circle represents a 16 ng dose norepinephrine injected at 6 min intervals. The mean of the last three responses before injection of serum (solid circles) was used as control value while the mean of the three highest consecutive responses after serum (solid circles) was used as the test value. This animal had a 7% increase in response after serum.

have been made in several studies involving both animals (10-13) and man (14-17). Other studies have yielded largely negative results (18-21). Much of the confusion surrounding this important facet of the hypertensive process stems from the nature of the experimental protocols used.

Investigations of vascular responsiveness using hypertensive animals themselves to assay for vasopressor response fail to distinguish between changes in apparent response, which can be largely due to geometric changes in resistance vessels and smooth muscle hypertrophy, and real



4. A comparison of the vasopressor response to norepinephrine in bioassay animals after the injection of normotensive and hypertensive serum. Bars represent the proportion of animals in activity within each serum group. Norepinephrine response after hypertensive serum was much higher than after normotensive serum. The median change in response after normotensive serum was 2 mm Hg, and was 12 mm Hg after hypertensive serum.

es in response that are due to increased sensitivity to vasopressor agents. These studies have utilized *in vitro* preparations such as helical strips of femoral artery and aorta (19-22) or portal veins of the investigators assuming that large vessels respond quantitatively to vasopressors in the same manner as the smaller resistance vessels.

The use of normotensive animals for the study of humoral vasopressor potentiators offers a number of advantages over *in vitro* techniques and methods that utilize hypertensive bioassay animals. Such a procedure avoids differences in reactivity that might be due to extrinsic or hypertrophic changes in the vascular bed brought about by the hypertensive process itself, and since the preparation is an *in vivo* system, it allows for expression of both direct and indirect effects of injected sera. In addition, there are fewer assumptions as how physiologic the assay system is. The effects observed are reflections of changes in the activity of the same determinants of total peripheral resistance and blood pressure that exist physiologically.

Recently, a study of Mizukoshi and Michelakis (8) reported a significant increase in vasopressor sensitivity to norepinephrine and angiotensin after the administration of small quantities of serum from hypertensive humans to an assay animal. This material was thought to be a heat-labile, nondialyzable substance that was secreted and released by the ischemic kid-

neys of patients with renovascular hypertension. It was also found in the plasma of patients with essential and malignant hypertension. In addition, sodium restriction in these hypertensive subjects unequivocally reduced the plasma's activity of this substance. It was thought that this substance might play some role in the development of hypertension.

In this study, the sera taken from rats made hypertensive by salt loading were investigated for the presence of a humoral factor that increases reactivity to the vasopressor effects of norepinephrine. Very small aliquots of serum from hypertensive animals were injected into normotensive, nephrectomized, ganglionically blocked rats, and the pressor responses to exogenous norepinephrine were compared to preinjection responses.

The injection of hypertensive sera into the bioassay animals led to a significant increase in vasopressor response to norepinephrine, an observation not made when normotensive sera was used. In contrast to the study of Mizukoshi and Michelakis (8), no change in basal mean blood pressure was observed. No overt attempt was made to characterize the nature or chemical properties of the humoral substance or substances involved, however, the material seems to be relatively heat stable, and its activity persists after repeated freezing and thawing. Studies to elucidate its chemical properties are now in progress.

In summary, the injection of very small aliquots of serum from hypertensive ani-

mals into bioassay animals increased the reactivity of these animals to the vasopressor effects of norepinephrine. These findings suggest the presence of a humoral factor or factors in the serum of hypertensive animals that augments their response to vasopressor agents and that may play some role in the hypertensive process.

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hypertensive Effect of Pregnancy in Spontaneously Hypertensive Rats (39471)

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Normal rat, blood pressure has been reported to decline during the last trimester of pregnancy (1, 2). Studies in human and dogs (4) have shown that this is accompanied by increased sodium excretion, presumably related to natriuretic factors in pregnancy (3). Normal rats do not demonstrate a preference for sodium until delivery (5). Thus sodium restriction needs and a decline in blood pressure appear to be associated with pregnancy in several species. The blood pressure lowering effect of sodium restriction can also be observed in rats made hypertensive by renal artery constriction with and without contralateral nephrectomy (6) or by wrapping the kidney in cellophane (perinephritis) (7). However, all forms of experimental hypertension are ameliorated by pregnancy, and as we have reported no change in blood pressure during pregnancy in rats with chronic DOCA-salt hypertension and unilateral nephrectomy.

Spontaneously hypertensive rats developed by Okamoto and Aoki have been extensively studied as a model of idiopathic hypertension. The present study was designed to examine the effect of pregnancy on blood pressure in a comparison to similar observations of the Kyoto-Wistar (KW)

Twenty-two female SHR and KW rats, 8 to 10 months of age, (Charles River Breeding Supply Company) were used. All rats were given free access to standard chow (Purina Company) and

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tap water. Half of the animals were used as nonpregnant controls. All females were mated with male rats of the same strain after vaginal smears indicated estrus. Males were caged with females for 2 nights and then removed following which the females were caged individually.

Systolic blood pressure was measured in the morning by a tail-cuff method (11). The rats were placed in an incubator at 37°C for 15 min and then placed in an adjustable container and the tail seated onto the plethysmograph. Three readings were averaged for each individual blood pressure determination. Pregnant animals were studied through delivery and for 2 days postpartum. Nonpregnant controls were studied throughout the same time period. Statistical analyses were performed by *t* test (12).

Results. Figure 1 shows systolic blood pressure in the nonpregnant SHR ($n = 12$) and normotensive KW rats ($n = 12$). As can be seen in the figure, blood pressure of nonpregnant SHR was significantly ($P < 0.001$) higher than that of nonpregnant KW rats.

Figure 2 similarly demonstrates systolic blood pressure in the pregnant SHR ($n = 10$) and pregnant KW ($n = 12$) rats. The pregnant SHR had significantly ($P < 0.005$) higher average systolic blood pressure than did pregnant KW rats during the initial 19 days of gestation, but demonstrated a marked decline in blood pressure from an average maximum of 201 ± 18 (SD) mm Hg to an average minimum of 123 ± 20 mm Hg during the 5 days before delivery. No significant difference in blood pressure was seen between the two groups on the day prior to delivery. The blood pressure in the pregnant KW rats was significantly lower ($P < 0.005$) on the delivery day, 113 ± 9 mm Hg than that in

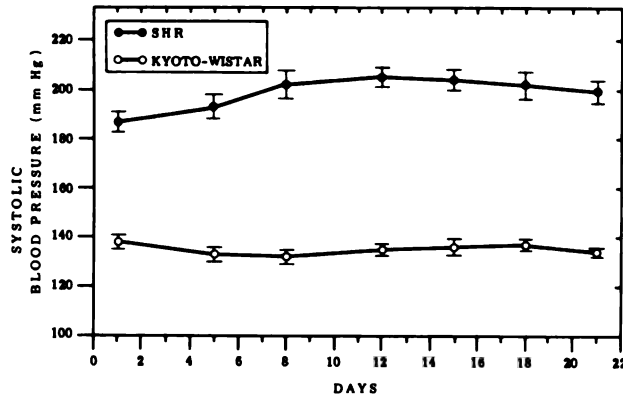


Fig. 1. Mean systolic blood pressure (± 1 standard error) in nonpregnant Kyoto-Wistar (open circles, $n = 12$), and SHR (closed circles, $n = 12$).

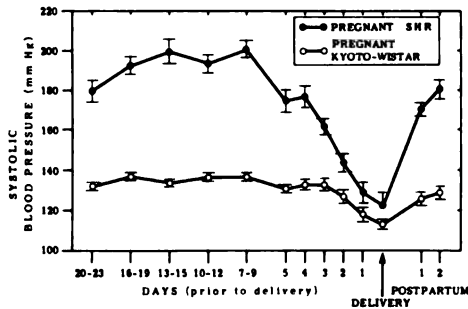


Fig. 2. Mean systolic blood pressure (± 1 standard error) in pregnant Kyoto-Wistar (open circles, $n = 12$), and SHR (closed circles, $n = 10$). The day of delivery is indicated by the arrow.

nonpregnant KW rats, 134 ± 6 mm Hg. Two days after delivery, blood pressure in pregnant SHR and KW rats again rose to an average level of 181 ± 5 and 129 ± 3 mm Hg, respectively.

Discussion. Although the SHR has been extensively studied as a model of spontaneous hypertension, the mechanisms for the development and maintenance of hypertension remain unclear. In the present study, the physiologic changes induced by pregnancy were associated with a marked decline in blood pressure to normal in the SHR during the last 3 days of gestation. Qualitatively similar changes in blood pressure were seen in the normotensive Kyoto-Wistar control rats. The mediation of these changes may be important in our understanding of the hypertension in the SHR.

The amelioration of hypertension by pregnancy is not unique to the SHR. Other forms of experimental hypertension in

rats, induced by renal artery constriction (6) or by bilateral renal encapsulation (7), have demonstrated marked decreases in blood pressure during pregnancy. However, not all forms of experimental hypertension are improved by pregnancy. Two studies (8, 9) have failed to observe a decrease in blood pressure in the animal made hypertensive by administration of DOCA and salt during pregnancy. The latter observations would imply that in the DOCA-salt model of experimental hypertension the natriuretic effects of pregnancy are not sufficient to reduce blood pressure. This may not necessarily be the case however, since it is recognized that the DOCA-salt model may have a hypertensive component unrelated to the acute effects of mineralocorticoid administration (meta-corticoid or post-DOCA hypertension) which persists after cessation of the steroid.

While the present study was restricted to observations of blood pressure, several possible mechanisms for the antihypertensive effect of pregnancy in the SHR can be speculated upon. Pregnancy is associated with a variety of humoral and hemodynamic changes. One example is the increased production of progesterone. Progesterone is known to be an antagonist of mineralocorticoid activity and thus a natriuretic factor. It has been reported that peak increases in plasma progesterone and its production rate occur between the 15th and 20th days of normal murine pregnancy (13, 14). It is curious that this is precisely the time when blood pressure began to

in the pregnant SHR of the present study. However, similar changes would be expected in the pregnant control rats. It has been reported that the non-pregnant SHR has significantly lower plasma aldosterone levels than Kyoto rats (15), it is conceivable that comparable levels of circulating progesterone have exerted a relatively greater natriuretic effect in the pregnant SHR. Thus it is possible that the marked decline in blood pressure observed in pregnant SHR may have been due to a relatively greater increase in extracellular fluid volume or a greater sensitivity to changes in volume in the pregnant normotensive rats.

It is also known that pregnancy is associated with increased production of prostaglandins, kinins, and other humoral factors thought to influence sodium and/or water metabolism. It is possible that the pregnant SHR, for unknown reasons, may be more sensitive to such factors. Indeed, we have speculated upon the role of angiotensin in abnormalities of blood pressure control during pregnancy (16). The observations of the present study do not permit precise identification of the factor responsible for the marked decline of blood pressure in the pregnant SHR. A number of mechanisms are possible. It has been demonstrated that pregnant animals and humans have greater sodium needs than their nonpregnant counterparts (3), and this is apparently due to a natriuretic effect of pregnancy (3). If this effect is sufficient to cause extracellular volume depletion, a reduction in blood pressure could result. Further studies of sodium and fluid balance are necessary to define whether these factors are responsible for the dramatic decrease in blood pressure in pregnant SHR observed in the present study. Alternatively, other factors may be involved in this phenomenon. Further investigation of the mechanisms responsible for the observations of the present study may clarify the etiology and pathophysiology of hypertension in the SHR.

Summary. Pregnancy in the SHR is associated with a marked and significant decline in blood pressure into the normal range before delivery. While a mild decrease in blood pressure was also seen during the last 3 days of gestation in the normotensive pregnant rats, the mechanism for the marked change in blood pressure in the SHR is not clear. Further investigation of the blood pressure effects of pregnancy in the SHR may improve our understanding of the pathophysiology involved in this model of experimental hypertension.

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Interference with the Serum Gastrin Response to Feeding after Surgical Intervent with the Rat Thyroparathyroid Complex¹ (39472)

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Previous work by us and by others has established that gastrin is a potent thyrocalcitonin secretagogue in a number of mammalian species, including man (1-4). Conversely, in man and other mammals some work has suggested that increased blood levels of thyrocalcitonin, in turn, can act to suppress a number of gastrointestinal functions, including secretion of gastrin (5-9).

Recently, we reported large increases in both serum gastrin and serum thyrocalcitonin shortly after the onset of feeding in rats adapted to a fixed schedule of daily feeding (10). Whether the increased secretion of thyrocalcitonin postprandially occurred in response to the increased level of circulating gastrin or to some other gastrointestinal factor or event remains to be clarified (10, 11). In the present study we used both rats adapted to a feeding schedule (10) and unadapted rats. The purpose of the experiments was to explore whether or not a decrease in blood thyrocalcitonin after thyroidectomy might lead to an increased blood level of gastrin indicating that thyrocalcitonin normally acts to suppress gastrin release. Surprisingly, the results showed that surgical intervention of any kind with the thyroparathyroid complex resulted in a suppression of the gastrin response to feeding.

Materials and methods. Animals. Male Holtzman rats 40-50 days old and weighing 150-200 g were adapted to a fixed schedule of daily feeding for 2 weeks or more as described previously (10). Food (Purina Laboratory Chow) was made available to each individually housed rat for 12 hr and

then withheld for 12 hr. The time of on feeding and of food removal were kept same (± 5 min) for each day. In order to ascertain possible effects of the light period (12 hr each), half of the rats were during the dark period and half during light period. However, since no differences were found between rats fed during the dark and those fed during the light, results from appropriate groups were combined for presentation. As described previously (10) consumption by rats during the first 2 hr of feeding was monitored to ensure that no significant differences occurred between the two groups being compared. Rats adapted to a feeding schedule were supplied Purina Laboratory Chow *ad lib.* for the 24 hr period before the experiment when they were fasted; after a 24 hr fast food was presented and the rats allowed to feed for the desired time interval. All rats were allowed water *ad lib.*

Surgical procedures. All surgery was performed under ether anesthesia. In order to allow time for resumption of normal feeding patterns, rats were not used for experiments until at least 2 days after surgery. Thyroparathyroidectomy, thyroidectomy, parathyroidectomy all were achieved by blunt dissection. Thyroidectomy was performed on rats which had previously been subjected to autotransplantation of parathyroid glands, and rats were supplemented with thyroxine as previously described (12). Parathyroid autotransplantation was achieved by surgically removing both glands and placing them either within the thyroid tissue or laterally to the thyroid in the sternohyoid muscle. Success of the transplantation procedure was determined for each rat 10-14 days after surgery by plasma calcium analysis as described previously (12). To interrupt vagal innervation to the stomach and still per-

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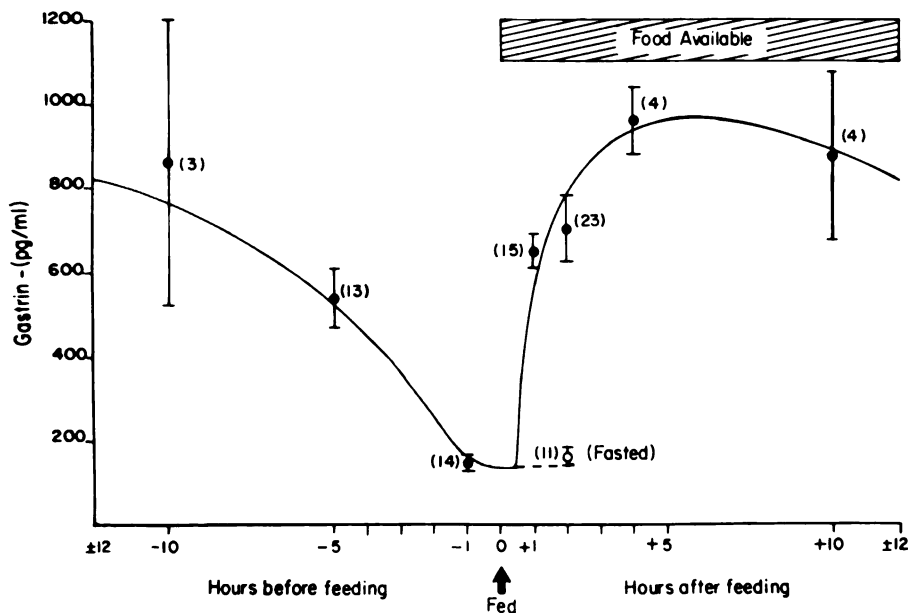
emptying, truncal vagotomy plus pyloroplasty were performed through a single abdominal incision. Both trunks of the vagus nerve were transected at the distal end of the esophagus. A 3 mm longitudinal incision was made at the pylorus through the muscle, and the incision was closed with nylon sutures. Control rats were subjected to pyloroplasty only.

Analyses. Radioisotopes (^{45}Ca and ^{32}P) were injected 1 week before blood samples were obtained. Blood was drawn from each rat by cardiac puncture and allowed to clot. Serum was obtained by centrifugation within 1 hr of blood collection. Analyses of calcium (13), phosphate (14), ^{45}Ca , and ^{32}P (10, 15) were performed on freshly obtained aliquots of the remaining serum sample from which the sample was stored at -20°C for subsequent immunoassay.

The procedures used for the radioimmunoassay for serum gastrin have been described previously in detail (16, 17). Equilibrium assays were conducted using a pig antiserum to porcine gastrin (titer = 1:100,000), and synthetic 1-

17 human gastrin (ICI, England) was used as unlabeled reference standard. Thus the values we report for gastrin in rat serum are in terms of pg-equivalents of synthetic human gastrin. Bound and free labeled gastrin were separated using amberlite resin (16, 17). The lower limit of detectability of the assay as conducted was 20 pg/ml, and intra- and interassay variations, as reported previously (10), did not exceed 15 and 20%, respectively.

Results. Figure 1 summarizes results obtained for serum gastrin in rats adapted to a fixed schedule of daily feeding. Previously we reported the serum gastrin changes in these rats during the period extending from 5 hr before feeding to 2 hr after the onset of feeding (10). Also reported were changes in calcium, phosphate, ^{45}Ca , ^{32}P , and thyrocalcitonin (10). The results in Fig. 1 extend our findings to cover the gastrin changes over a 24 hr period. Serum gastrin levels were measurable at all time periods studied, reaching a nadir 1 hr before feeding (~ 150 pg/ml), rising rapidly more than fivefold 1-2 hr after the onset of feeding and remaining at levels >500 pg/ml for most of the daily



1. Cyclic change in serum gastrin over 24 hr in intact rats adapted to a fixed schedule of daily feeding, as expressed in relation to the onset of feeding (= 0 time). Each point represents the mean value for a group whose number is shown in parentheses; vertical lines show the SE. Open circle and dashed line represent a rat whose food was withheld at 0 time. Light-dark schedules were adjusted so that some rats were fed during light and others fed during dark (see Methods).

period before the next scheduled feeding. Serum gastrin remained low 2 hours after the scheduled feeding period if food was withheld. The postprandial increase in gastrin was not associated with an increase in blood calcium, since this is when daily serum calcium is lowest in adapted rats (10). Furthermore, as we have noted previously (10), serum gastrin levels were unusually high in these "adapted" rats. Even the lowest value obtained (~ 150 pg/ml) was higher than values obtained 1 hr after feeding in most nonadapted rats (Table I). The results shown in Table I are representative of our findings in several experiments with nonadapted rats, namely that serum gastrin was undetectable (<20 pg/ml) in rats fasted for 20 hr or longer and that subsequent feeding raised serum gastrin to only about 100–150 pg/ml 1–2 hr later.

The results shown in Table II illustrate that the pattern of gastrin response in rats adapted to the regular feeding schedule was not abolished by disruption of the normal vagal influence on gastrin release. As reported previously by others (18), we found that vagotomized rats exhibit an elevated serum level of gastrin before and after feed-

ing compared to control rats which, in this experiment, had been subjected to pyloroplasty alone. The rats with pyloroplasty alone exhibited serum gastrin levels which were not different from unoperated rats (see Fig. 1) at the time periods studied.

Figure 2 summarizes the serum gastrin changes in rats adapted to the daily feeding schedule and subjected to surgical intervention with the thyroparathyroid complex. At the time periods examined just before and after feeding, thyroparathyroidectomized, parathyroidectomized, and thyroidectomized (with autotransplanted parathyroids) rats all showed marked reductions in fasting gastrin levels and a severely impaired gastrin response to feeding. Rats with intact thyroid glands and autotransplanted parathyroid glands exhibited a delayed rise (at 1 hr) and less marked (at 2 and 6 hr) suppression of serum gastrin. In addition to a reduced response to feeding, these groups of rats also appeared to have a delayed response, since unlike in intact control rats no significant rise in serum gastrin was observed at 1 hr after the onset of feeding. In a few rats (Fig. 2—sham) the parathyroid glands were excised and then replaced either in the normal location on the surface of the thyroid or within the thyroid tissue. These rats (seven at each time period), adapted to the fixed feeding schedule, showed serum gastrin levels of 671 ± 124 pg/ml 1 hour after feeding, and 839 ± 287 pg/ml 2 hr after feeding. These preliminary findings suggest but do not establish that a close approximation of the parathyroid and thyroid glands is required to prevent the reduced gastrin response observed after surgical intervention with the thyroparathyroid complex.

In order to determine whether the re-

TABLE I. SERUM GASTRIN IN FASTED AND FED RATS NOT ADAPTED TO A FIXED SCHEDULE OF DAILY FEEDING.

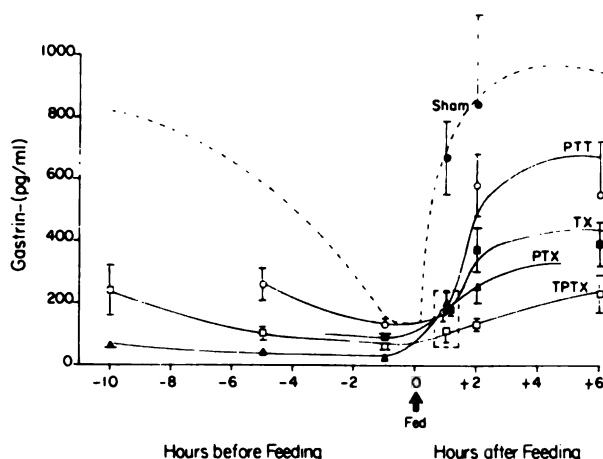
Treatment	Serum gastrin pg/ml
1. Fasted 24 hr (4)	N.D. ^a
2. Fasted 24 hr + fed 1 hr (9)	89 ± 23

^a N.D. = Not detectable (value for each rat was <20 pg/ml). Fed rats were offered Purina Laboratory Chow for 1 hr after a 24 hr fasting period. Each rat consumed 4–6 g. Serum gastrin was measurable in each rat in group 2. Serum Ca (mg/dl) for each rat was between 10.0 and 10.5. Numbers in parentheses represent the number of rats in each group.

TABLE II. SERUM GASTRIN IN VAGOTOMIZED RATS ADAPTED TO A FIXED SCHEDULE OF DAILY FEEDING.

Treatment	Serum gastrin pg/ml			
	-5 Hr	-1 Hr	+1 Hr	+2 Hr
1. Pyloroplasty	401 ± 111 (4)	N.T. ^a	558 ± 94 (6)	561 ± 106 (4)
2. Pyloroplasty + Vagotomy	1108 ± 318 (4)	297 ± 52 (4)	N.T.	1619 ± 274 (5)

^a N.T. = Not tested. Times shown are expressed relative to the time of onset of feeding (0 hr) when rats were offered Purina Laboratory Chow. Each rat consumed 4–6 g. Numbers in parentheses represent the number of rats in each group. Daily plasma calcium was within the range for normal rats at each hour tested.



Cyclic changes in serum gastrin in rats adapted to a fixed schedule of daily feeding and subjected to intervention with the thyroparathyroid complex. Surgical procedures employed were "Sham" (parathyroid removed and replaced in thyroid), PTT (parathyroids autotransplanted in neck muscle), TX (thyroidectomy), PTX (thyroids autotransplanted to neck muscle), PTX (parathyroidectomy), and TPTX (thyroparathyroidectomy at +1 hr emphasizes the reduced serum gastrin in all groups except "Sham" with parathyroids in cation. Numbers of animals at -1 hr to +2 hr = 9-23; all other points represent 3-12 animals. Dashed line represents curve for normal rats shown in Fig. 1. See Fig. 1 legend for additional details. Plasma calcium levels with functional parathyroid glands were >10 mg/dl at -5 hr; for rats without parathyroids plasma levels were between 7 and 8 mg/dl.

gastrin response to feeding in the thyroid transplanted rats conditioned to a fixed schedule (Fig. 2) was somehow different from rats not adapted to this feeding schedule. We also were studying the effect of thyroparathyroid surgery on serum gastrin 2 hr after feeding (Table III). Rats adapted only to parathyroid autotransplantation showed an increase in blood gastrin similar to that seen in nonadapted intact rats (Table I). However, rats which had been thyroidectomized for 48 hr showed no detectable increase in serum gastrin after feeding.

To explore further the apparent difference in the serum gastrin response to feeding in the adapted rats subjected to surgery (Fig. 2), the study summarized in Fig. 3 was conducted. Changes in plasma calcium, inorganic phosphate, ^{45}Ca , and ^{32}P as well as gastrin were evaluated in intact and parathyroid transplanted rats from 1 hr before feeding to 2 hr after feeding. In this experiment both parathyroid glands were excised and transplanted laterally in the neck. Except for gastrin, values are shown as percent from the plasma concentration observed 1 hr before feeding. Plasma calcium

TABLE III. SERUM GASTRIN IN THYROIDECTOMIZED AND THYROID INTACT RATS WITH AUTOTRANSPLANTED PARATHYROID GLANDS AND NOT ADAPTED TO A FIXED SCHEDULE OF DAILY FEEDING.

Treatment	Serum gastrin pg/ml
1. Parathyroid transplanted	
a. Fasted 24 hr	N.D. ^a
b. Fasted 24 hr + fed 2 hr	88 ± 22
2. Parathyroid transplanted + thyroidectomized	
a. Fasted 24 hr	N.D.
b. Fasted 24 hr + fed 2 hr	N.D.

^a N.D. = Not detectable (value for each rat was <20 pg/ml). Fed rats were offered Purina Laboratory Chow for 2 hr after a 24 hr fasting period. Each rat consumed 5-10 g. Plasma Ca values for each group averaged between 10.1 and 10.3 mg/dl (five rats/group).

is not shown in Fig. 3 because by 1 hr before feeding, as previously reported (10), plasma calcium had already fallen in both groups and did not change further at either +1 hr or +2 hr. Serum gastrin levels in the rats with parathyroids transplanted away from the thyroid did not differ significantly from those in intact rats 2 hr after feeding. However, at +1 hr the transplanted rats exhibited a significantly lower serum gastrin

than intact rats. Likewise, the transplanted rats showed either a delayed or reduced change at +1 or +2 hr in serum phosphate, ^{32}P , and ^{45}Ca (Fig. 3).

Discussion. Several recent studies have suggested that in man and certain other species high levels of blood thyrocalcitonin may act to suppress gastrin secretion and a variety of other gastrointestinal functions (5-9). However, these studies generally have employed large doses of administered calcitonins (8, 9). Because of this, no strong evidence exists to support the idea that physiological levels of circulating thyrocalcitonin influence secretion of gastrin.

This study was originally designed to determine whether or not removal of endogenous thyrocalcitonin by thyroidectomy might lead to an elevation in blood gastrin. We relied heavily on studies in rats adapted to a specific feeding schedule (Fig. 1), because these rats exhibit postprandial increases in gastrin and fasting serum gastrin levels which are much greater than those in nonadapted rats (Tables I and III) and which become even more pronounced after truncal vagotomy (Table II). Unexpectedly,

we found that thyroidectomy produced a delayed and reduced increase in serum gastrin instead of an increase (Fig. 2) and that this occurred also in rats that had not been adapted to a daily feeding schedule (Table III). Further studies revealed that any surgical intervention which disrupted the normally close apposition of the thyroid and parathyroid glands reduced the basal serum gastrin and delayed the usual increase in gastrin after feeding (Fig. 2). The reasons for the reduced and/or delayed rise in gastrin are unclear at present. A low blood calcium in PTX and TPTX rats might be involved since gastrin release is known to be influenced by serum calcium (1, 19, 20). Alternatively, parathyroid hormone may have some direct effect on gastrin secretion (21-23). However, a reduced serum gastrin was observed also in thyroidectomized rats with parathyroid transplants having a normal serum calcium and thyroxine supplement (Fig. 2); presumably these rats were deficient in thyrocalcitonin. Even rats with both parathyroid and thyroid glands intact but with the glands separated anatomically showed a reduced and delayed rise in gastrin

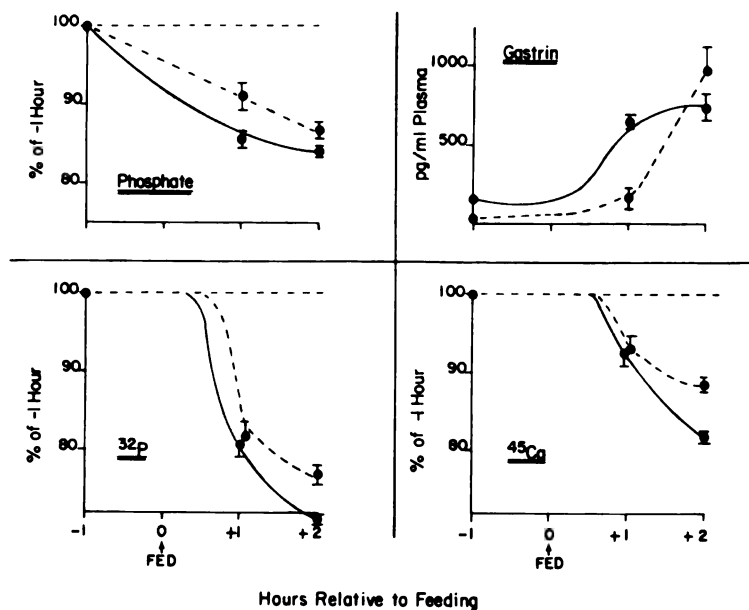


FIG. 3. Changes in serum gastrin, phosphate, ^{32}P , and ^{45}Ca in intact rats (—●—) and parathyroid autotransplanted (PTT) rats (---●---) adapted to a fixed schedule of daily feeding. Parathyroid glands were autotransplanted laterally into neck muscles. Except for gastrin, the results are expressed as relative change of each group from the value observed 1 hr before feeding. Plasma calcium and phosphate values at the -1 hr period were not statistically different for the two groups. See Fig. 1 legend for additional details.

ting (Fig. 2, PTT vs Control). In y treated rats, delayed or reduced i in phosphate, ^{32}P , and ^{45}Ca also observed (Fig. 3). Interestingly, rats their own parathyroids autotrans-back within or on the surface of the showed a normal rise in blood gas-r feeding. The results show that the and parathyroid glands are required nal gastrin secretory responses after and suggest that the normally close ical apposition of these two glands of some special significance.

ary. Rats adapted for 2-3 weeks to schedule of daily feeding exhibited um gastrin levels and large increases n gastrin after feeding. Serum gastrin 00 pg/ml throughout most of the 24 r cycle and fell to ~150 pg/ml 1 hr he scheduled time of feeding; by 1-2 feeding serum gastrin rose again to g/ml. This postprandial increase in was not observed if food was with-d it was seen also after truncal vagot-hich itself raised serum gastrin. In t, in nonadapted rats serum gastrin easurable (<20 pg/ml) after a 24 hr d rose only to ~100 pg/ml 1-2 hr eding. In nonadapted rats, thyroid-prevented the 2 hr rise in serum after feeding. In adapted rats para-ectomy, thyroparathyroidectomy, roidectomy with parathyroid auto-ntation, all reduced serum gastrin and after feeding indicating that any intervention with the thyroparathy-nplex interfered with gastrin release. vith autotransplanted parathyroid and intact thyroid glands showed a l increase in serum gastrin after feed-well as a delay in the usual fall in phosphate, ^{45}Ca , and ^{32}P , but if the roids were replaced within the thy-sue, this delay did not occur.

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Dynamics of Luteinizing Hormone Release after Intravenous Administration of Crude Stalk-Median Eminence Extract or Synthetic Gonadotropin-Releasing Hormone¹ (39473)

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The ability of acidic extracts of basal medial hypothalamic nuclei to induce the release of gonadotropins has been well documented (1-3). More recently a compound of hypothalamic origin capable of releasing gonadotropins has been isolated (4), identified (5), and chemically synthesized (6). This compound, gonadotropin-releasing hormone (Gn-RH) has been the subject of much intensive investigation in recent years. However, the dynamics of luteinizing hormone (LH) release following injection of Gn-RH (7-9) appear to be quite different than the release observed after injection of crude stalk-median eminence (SME) extracts (10, 11). Similar discrepancies have been observed following the *in vitro* perfusion of anterior pituitaries with partially purified hypothalamic extracts (12) or with crude hypothalamic extracts (13). Thus, the possibility existed that crude hypothalamic extracts contained substances, other than Gn-RH, which were capable of inducing the release of LH.

The following investigations were designed to: (i) compare the dynamics of LH release after injections of crude SME extracts or synthetic Gn-RH, and (ii) determine if hypothalamic factors other than Gn-RH contributed significantly to the release of LH.

Materials and methods. Mature Western range ewes in seasonal anestrus or that had been ovariectomized from 3 to 60 days were used. An indwelling polyvinyl cannula with a Teflon obturator (Safedwell, Becton-Dickinson, Co., Rutherford, New Jersey) were inserted into the jugular vein of each ewe for injections of test materials and for collection of blood samples. Crude extracts of porcine SME prepared as described by

Schally *et al.* (14) and synthetic Gn-RH was generously supplied by Abbott Laboratories, North Chicago, Illinois.

Radioimmunoassay. All serum samples were assayed for LH and Gn-RH as described previously (15, 16). Acidic extracts of SME were neutralized with 1 *N* ammonium hydroxide, lyophilized, and reconstituted in phosphate-buffered saline containing 0.1% gelatin and 0.1% sodium azide (gel-PBS). The LH and Gn-RH content of these extracts were determined by assaying from 5×10^{-1} to 5×10^{-7} SME equivalents.

Experiment I. Four ewes which had been ovariectomized for 3 days were given an intravenous (iv) injection of 4 SMEs or 10 μ g synthetic Gn-RH according to the schedule in Table I. Jugular blood samples were collected at -45, -30, -15, 0, 3, 6, 9, 12, 15, 18, 24, 30, 36, 42, 48, 54, 60, 72, 84, 96, 108, 120, 150, 180, 210, and 240 min relative to the administration of the material to be tested. The samples were placed in an ice-water bath immediately after collection and were allowed to clot overnight. Serum was separated by centrifugation and stored frozen until assayed for LH and Gn-RH.

Experiment II. Four ewes which had been ovariectomized for 60 days were given an iv injection of 4 SME equivalents or 300 ng synthetic Gn-RH in a design similar to that for Experiment I. Jugular blood samples were collected and processed as described for Experiment I.

The results of Experiments I and II indicated that the dynamics of LH release were different after administration of crude SME extract than after administration of synthetic Gn-RH. Radioimmunological analysis of the crude SME extract indicated a contamination of 15.8 μ g LH per SME equivalent. Therefore, ammonium sulfate precipitation and gel-filtration were used to

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the quantity of LH in the crude SME

Experiment III. Four anestrus ewes were given an iv injection of 4 SME equivalents. The SME equivalents which had been purified by precipitating the large protein with 50% saturated ammonium sulfate. The injection schedule and collection and processing of blood samples were similar to that described for Experiment I.

Experiment IV. Twenty SME equivalents were purified by gel-filtration on a 1×30 column of Sephadex G-25 eluted with 0.1 M acetic acid. Ten microliters of each fraction were diluted to 1 ml with gel buffer. Duplicate 200 μ l aliquots of the fractions were assayed for LH and Gn-RH. Fractions containing immunoreactive Gn-RH were discarded. The remaining fractions were neutralized with 1 N ammonium hydroxide, lyophilized, and reconstituted in physiological saline. Four anestrus ewes were given iv injections of 4 crude SME equivalents or 4 SME equivalents purified by gel-filtration. The injection schedule and collection and processing of blood samples is described in Experiment I.

Results. The results from Experiment I are depicted in Figs. 1 and 2. Following the injection of 4 SME equivalents there was a moderate rise in levels of Gn-RH and

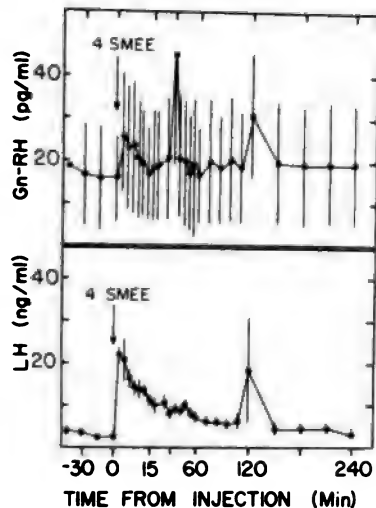


FIG. 1. Levels of Gn-RH and LH in serum of ewes following iv administration of 4 crude stalk-median equivalents (SMEE). Each point represents the mean \pm standard error ($n = 4$).

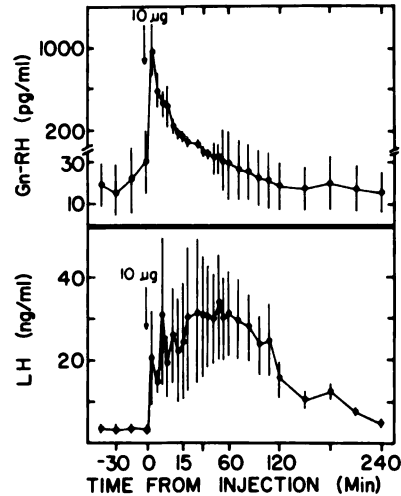


FIG. 2. Levels of Gn-RH and LH in serum of ewes following iv administration of 10 μ g of synthetic Gn-RH. Each point represents the mean \pm standard error ($n = 4$).

TABLE I. EXPERIMENTAL DESIGN FOR INJECTION OF SME EXTRACT OR SYNTHETIC Gn-RH INTO EWES

Treatment	Day 1	Day 2
SME	Ewe 1 Ewe 3	Ewe 2 Ewe 4
Gn-RH	Ewe 2 Ewe 4	Ewe 1 Ewe 3

LH in serum. Both hormones reached a peak (22 ± 3 ng/ml LH and 27 ± 13 pg/ml Gn-RH) within 3 min and then began to decline. Levels of Gn-RH had returned to baseline within 15 min whereas levels of LH did not return to baseline for 60 min after the injection of SME extract (Fig. 1). The increased levels of Gn-RH in samples collected at 30 and 120 min after injection were due to high levels in a single animal. The increase of Gn-RH noted at 120 min was associated with an increase in serum levels of LH in the same ewe. After an iv injection of 10 μ g synthetic Gn-RH into the same ewes, levels of Gn-RH and LH in serum followed a different pattern. Levels of Gn-RH reached a peak within 3 min and declined to basal levels within 90 min (Fig. 2). The magnitude of the Gn-RH increase obtained after injection of 10 μ g synthetic Gn-RH was 964 ± 216 pg/ml compared to an increase of 10.6 ± 2.2 pg/ml following the injection of 4 crude SME equivalents. The magnitude of the LH response follow-

ing the different treatments was similar (22.4 ± 0.5 ng/ml after administration of 4 crude SME equivalents compared to 33.6 ± 12.1 ng/ml after 10 μ g synthetic Gn-RH). Although levels of LH began to increase immediately after injection of either Gn-RH or crude SME extract the time required to obtain maximal concentrations of LH was quite different (3.8 ± 0.6 min after crude SME vs 57.4 ± 13.3 min after synthetic Gn-RH). Furthermore, levels of LH remained elevated much longer after injection of synthetic Gn-RH.

The content of Gn-RH in crude SME extract was 68 ng/SME equivalent in Experiment II. The dosage of synthetic Gn-RH injected was similar to the amount of Gn-RH contained in 4 SME equivalents. After injection of 4 SME equivalents, levels of Gn-RH in serum increased 38 ± 8 pg/ml within 3 min and returned to baseline within 15 min. Levels of LH in serum increased 25.0 ± 8.2 ng/ml within 9 min and had returned to baseline within 150 min (Fig. 3). Following the injection of 280 ng synthetic Gn-RH the increase in levels of Gn-RH in serum was similar to that observed after injection of 4 crude SME equivalents (Fig. 4). However, the increase in levels of LH in serum (6 ± 3 ng/ml) was much smaller. The interval from injection until maximum levels of LH were observed averaged 4.5 ± 1.5 min after injection of crude

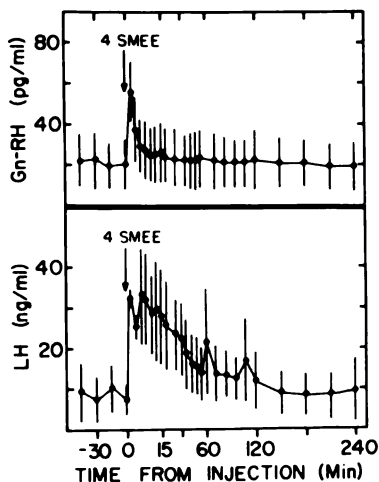


FIG. 3. Levels of Gn-RH and LH in serum of ewes following iv administration of 4 crude SMEE. Each point represents the mean \pm standard error ($n = 4$).

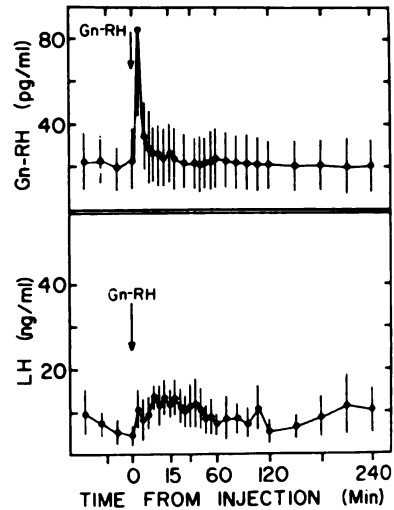


FIG. 4. Levels of Gn-RH and LH in serum of ewes following iv administration of 280 ng synthetic Gn-RH. Each point represents the mean \pm standard error ($n = 4$).

SME compared to 9.7 ± 3.6 min after injection of synthetic Gn-RH.

In experiment III iv injection of 4 crude SME extracts into anestrus ewes resulted in increases in levels of LH and Gn-RH similar to those observed after injections of crude SME in Experiments I and II. Injection of crude SME extracts which had been treated with 50% saturated ammonium sulfate resulted in increases in levels of Gn-RH and LH similar to those observed after injection of 280 ng synthetic Gn-RH (Fig. 5).

In Experiment IV levels of Gn-RH and LH following injections of crude SME extract were similar to those observed in previous experiments. Levels of Gn-RH in serum after injection of crude SME extract which had been purified partially by gel filtration were similar to levels observed after administration of crude SME extract or after administration of 280 ng synthetic Gn-RH. The increase in the concentration of LH noted in these ewes was similar to that observed after injection of crude SME extract which had been treated with 50% saturated ammonium sulfate or after the injection of 280 ng synthetic Gn-RH (Fig. 6).

Discussion. Since the identification of Gn-RH and subsequent synthesis of Gn-RH, several reports have suggested that other compounds of hypothalamic origin are capable of releasing LH.

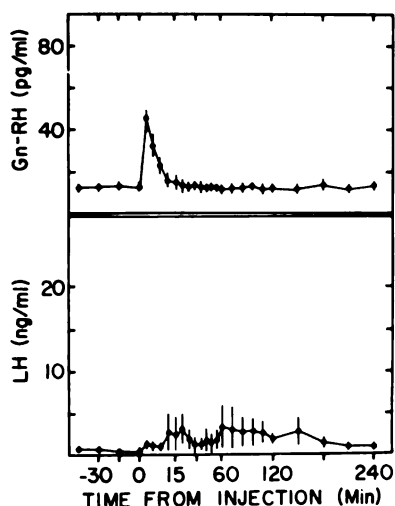


FIG. 5. Levels of Gn-RH and LH in serum of ewes following iv administration of 4 SMEE which were partially purified by ammonium sulfate fractionation. Each point represents the mean \pm standard error ($n =$

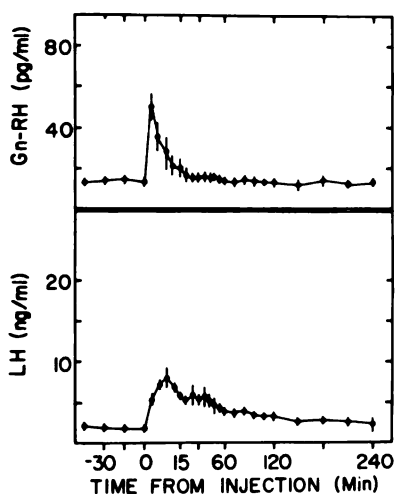


FIG. 6. Levels of Gn-RH and LH in serum of ewes following iv administration of 4 SMEE partially purified by gel-filtration. Each point represents the mean \pm standard error ($n = 4$).

gonadotropins (17-19). Differences in the dynamics of LH release following injections of crude SME extract or Gn-RH seemed to support this concept. Furthermore, data obtained by Spies *et al.* (11) suggested that crude SME extracts induced release of LH much more effectively than Gn-RH in rhesus monkeys (9, 20). Data obtained from Experiments I and II of this

study suggested that crude extracts of porcine SME were more effective in causing the release of LH in ewes than was synthetic Gn-RH. However, when the crude SME was partially purified by treatment with 50% saturated ammonium sulfate or by gel-filtration the releases of LH elicited by the SME extracts, or by a similar amount of synthetic Gn-RH, were similar. Subsequent analysis of the crude SME indicated that the factor resulting in increased levels of LH in serum that could be separated from Gn-RH by gel-filtration or by ammonium sulfate fractionation was, in fact, LH. It is possible that another factor capable of releasing LH was separated from Gn-RH by ammonium sulfate precipitation and by gel-filtration. However, this does not seem likely since the quantity of LH contamination in the crude SME extracts accounted for more than 90% of the increase in levels of LH in serum following injection of these substances, based on a volume of distribution for LH of 3.5 liters in the sheep (21).

That the release of LH after an injection of partially purified SME extracts or an equal amount of synthetic Gn-RH was similar (Experiments III and IV) suggests that Gn-RH is the only substance present in crude SME extracts in quantities sufficient to induce the release of LH following intravenous injections into sheep. This is further substantiated by reports indicating that both active and passive immunization against Gn-RH results in gonadal atrophy in rabbits (22), rats (23, 24) and sheep (Nett *et al.*, unpublished observation).

It seems likely that the rapid appearance of LH in the blood stream following injection of crude SME extracts reported by other investigators (10, 11) could have been the result of LH contamination in their extracts. Although Gay *et al.* (10) made no attempt to remove LH from their crude SME they reported that contamination accounted for less than 1% of the increase in serum levels of LH. Spies *et al.* (11) observed identical responses to crude SME and "LH-absorbed" SME. Similarly, when crude rat hypothalamic extract was used to superfuse rat hemipituitaries the release of LH was rapid and dramatic (13) even after correction for measurable LH contamination.

tion in the extract. In contrast, the release of LH from superfused bovine pituitary tissue was slight (12) when substances having a molecular weight of greater than 10,000 were removed from acidic extracts of bovine hypothalami by ultrafiltration.

The data obtained in this investigation and the available literature suggest that hypothalamic extracts contain substances of a molecular weight greater than 10,000 which increase immunoreactive levels of LH *in vivo* and in the medium used to perfuse pituitaries. In the present study this material appeared to be immunologically active LH.

From the levels of Gn-RH obtained after systemic injection of 10 μ g Gn-RH or 280 ng Gn-RH and the resulting release of LH after these injections it is suggested that levels of Gn-RH in the hypothalamo-hypophyseal portal circulation of the ewe must increase to between 60 and 940 pg/ml, or remain elevated above baseline from 15 to 60 min, to induce a substantial release of LH. Direct measurement of the Gn-RH in portal blood collected from monkeys (25, 26) on the day of the expected LH surge indicated an increase in levels of Gn-RH to approximately 500 pg/ml. Similar data have been obtained in rats (27).

Summary. The comparative ability of crude acidic extracts of stalk-median eminence (SME) and of synthetic Gn-RH to induce the release of LH in ewes was examined. Intravenous injections of 4 SME equivalents resulted in a rapid increase in serum levels of LH ranging from 27 to 54 ng/ml and with maximal levels occurring from 3 to 6 min postinjection. Injection (iv) of 10 μ g synthetic Gn-RH resulted in a prolonged release of LH into the circulation with serum levels of LH ranging from 21 to 49 ng/ml and with maximum levels occurring from 36 to 84 min postinjection. Administration of 280 ng synthetic Gn-RH (the quantity of Gn-RH contained in 4 SME equivalents) resulted in only a slight increase in serum LH (6 ± 3 ng/ml) with the maximum level at 9.7 ± 3.6 min post-injection. Injection of crude SME extracts after partial purification by ammonium sulfate precipitation or by gel-filtration resulted in only slight increases in serum levels of LH. Radioimmunoassay of the crude SME ex-

tracts indicated that over 90% of the increase in serum levels of LH observed after injection of crude SME could be accounted for by contamination of LH in the original extract.

Levels of Gn-RH in serum increased 10 to 60 pg/ml within 3 min after iv injection of crude SME extract or 280 ng synthetic Gn-RH and returned to baseline within 15 min. After iv administration of 10 μ g synthetic Gn-RH serum levels of Gn-RH increased to 964 ± 216 pg/ml and returned to baseline within 60 min. Since a substantial release of endogenous LH occurred only after administration of 10 μ g synthetic Gn-RH it is suggested that levels of Gn-RH in the hypophyseal portal circulation must approach 900 pg/ml to induce an increase in serum levels of LH comparable to the preovulatory surge.

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Differential Effects of *p*-Chloromercuriphenyl Sulfonate on Arterial Responses to Epinephrine and Serotonin¹ (39474)

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The purpose of this investigation was to study the effects of *p*-chloromercuriphenyl sulfonate (PCMBS), a potent sulfhydryl (SH) binding agent (1-6), on contractile responses elicited with epinephrine and serotonin in isolated preparations of arterial smooth muscle. In this way, we could examine whether or not SH groups are importantly involved in agonist-receptor systems for vasoactive stimulants.

It is generally stated that vascular receptor-sites for vasoactive agents such as acetylcholine, epinephrine, and serotonin consist largely of proteins (7-9). The importance of SH groups in maintaining biological activity has been well established for a variety of protein structures including enzymes (10), membrane-bound transport moieties (1-4), and steroidal (5) and cholinergic receptors (6). However, the extent to which SH groups represent important functional groups of receptors for epinephrine or serotonin in arterial smooth muscle is unknown. Such information is of interest because chemical characterization of vasoactive receptor-sites could provide insight into the mechanistic basis of agonist-receptor interactions.

Methods. Sprague-Dawley rats of either sex (350-450 g) were anesthetized with ether and decapitated. Helical strips of arterial smooth muscle measuring about 4 to 6 mm long, 400 μ m wide and 1 mm thick were prepared from the ventral tail artery as described previously (11).

Four arterial strips (two from each of two rats) were mounted in a single muscle chamber. One end of each strip was fixed to a separate post while its other end was attached to an isometric force transducer

(Grass, FT-03) by means of a silk thread. The strips were stretched to optimal length by imposing a resting tension of 500 mg and equilibrated for 2 hr in 20 ml of physiological salt solution (PSS) which was maintained at 37°C and gassed continuously with 95% O₂ and 5% CO₂ (11). The pH of the PSS was 7.25 to 7.35, and its composition (mmole) was NaCl 130, KCl 4.7, KH₂PO₄ 1.18, MgSO₄·7H₂O 1.17, CaCl₂ 1.6, NaHCO₃ 14.9, CaNa₂ versenate 0.026, and dextrose 5.5.

Serotonin creatinine sulfate (Mann Labs) or the racemate mixture of epinephrine-HCl (Parke-Davis) was dissolved in PSS and added (0.2 ml) to the tissue bath. The final concentration (10^{-5} M) of each agonist tested produced maximal increases in isometric tension. However, the concentration of serotonin represents a maximal dose, whereas the concentration of epinephrine was supramaximal (11).

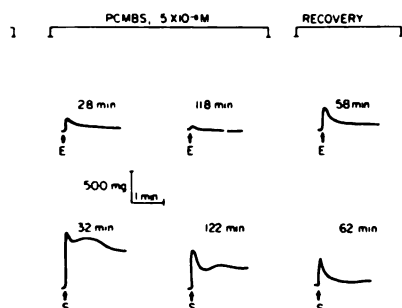
Responsiveness of the muscle strips to several challenges with epinephrine and with serotonin was examined at the beginning of each experiment. The maximal tension generated during a 2 min exposure to the agonist tested was designated as the control response. After each challenge with agonist, the muscle chamber was rinsed three times with PSS so that tension returned to baseline levels.

The bathing medium was replaced with PSS supplemented with PCMBS (Sigma, 10^{-6} to 10^{-4} M). Only one concentration of PCMBS was examined in each experiment. Challenges with agonists were tested after about 10, 30, 60, and 120 min of contact with PCMBS. Preliminary experiments performed in the absence of PCMBS showed that repeated responses to either serotonin or epinephrine were reproducible over this period of incubation. When the last challenge was completed, the chamber was

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0 min intervals with PCMBS-free PSS strips were again challenged to recover of responsiveness. Responses obtained in the presence of PCMBS, as well as those obtained during the recovery period, were expressed as a percentage of the control response. Statistical significance of differences between responses reproduced by each agonist in the presence and absence of PCMBS was assessed by Student's *t* test.

To determine whether or not cysteine, and not the SH group, produced the inhibition of responses to epinephrine (10⁻³ M) and PCMBS were added to the muscle chamber in three different experiments.



Contractile responses to epinephrine and serotonin in a single arterial strip are shown before, during incubation with PCMBS, and after replacing the strip with PCMBS-free PSS. Arrows indicate time of agonist into the muscle chamber. Note that the response to serotonin was maintained (moderate inhibition of contact with PCMBS) while the response to epinephrine was markedly inhibited.

The effects of alpha-adrenergic blockade with 10⁻⁴ M phentolamine (Ciba) on responsiveness to epinephrine and serotonin was examined in eight different tail artery strips prepared from four rats. Responses were evaluated before and 30 min after incubation with phentolamine. Similar experiments were performed with six additional preparations (three rats) in the presence and absence of 5 × 10⁻⁶ M phentolamine.

To test for the presence of beta-adrenergic receptors, responses to DL-isoproterenol (MCB, 10⁻⁹ to 10⁻⁵ M) were evaluated in eight preparations from four rats in the presence and absence of phentolamine (5 × 10⁻⁶ M).

Results. Maximal isometric tension generated by rat tail artery smooth muscle in response to 10⁻⁵ M epinephrine (1341 ± 104 mg) was similar to the tension generated in response to 10⁻⁵ serotonin (1296 ± 107 mg).

PCMBS consistently produced time and dose-dependent inhibition of contractile responses elicited with either epinephrine or serotonin (Figs. 1, 3, Table I). In other words, inhibition of responsiveness became progressively more pronounced as the duration (10–120 min) of incubation with each concentration of PCMBS was lengthened. Similarly, the extent of inhibition attained after a given period of incubation with PCMBS increased markedly as the concentration (10⁻⁶ to 10⁻⁴ M) of the sulfhydryl binding agent was increased.

However, inhibition of responses to epinephrine always was dramatically more pro-

TABLE 1. CONTRACTILE RESPONSES TO EPINEPHRINE AND SEROTONIN IN THE PRESENCE OF PCMBS.^a

Agonist	Time of PCMBS (min)	Contractile response (% of control)		
		Concentration of PCMBS (M)		
		10 ⁻⁶	7.5 × 10 ⁻⁶	5 × 10 ⁻⁵
Epinephrine (10 ⁻⁵ M)	10	96 ± 4	71 ± 2	1 ± 0.4
	30	94 ± 4	37 ± 4	0.7 ± 0.4
	60	90 ± 3	9 ± 5	0
	120	82 ± 4	2 ± 1	0
Serotonin (10 ⁻⁵ M)	10	96 ± 2	82 ± 2	73 ± 3
	30	92 ± 4	76 ± 2	75 ± 4
	60	89 ± 5	70 ± 3	58 ± 4
	120	88 ± 2	62 ± 4	60 ± 2

Helical strips of tail artery smooth muscle (four rats) were studied with each concentration of PCMBS (10⁻⁶ to 10⁻⁴ M). Numbers appearing below each concentration of PCMBS show the response (% of control) to epinephrine or serotonin (mean ± 1 SE). PCMBS markedly inhibited responses to epinephrine but responses to serotonin were depressed to a smaller extent.

nounced than the inhibition of responses to serotonin. Thus, the contractile response to epinephrine was markedly reduced to $41 \pm 2\%$ of control (59% inhibition, Fig. 2B) after a 10 min exposure to an intermediate concentration ($10^{-5} M$) of PCMBS, whereas the response to serotonin was only decreased to $86 \pm 2\%$ of control (14% inhibition, $P < 0.001$). Furthermore, the response to epinephrine was virtually abolished after 120 min of incubation with PCMBS while the response to serotonin had decreased by only about 50% ($P < 0.001$). Indeed, complete inhibition of epinephrine-induced contractions was achieved regularly with concentrations of PCMBS ranging from $5 \times 10^{-6} M$ to $10^{-4} M$ (Fig. 2, Table 1). In contrast, serotonin-induced contractions were always demonstrable at each concentration of PCMBS tested.

Considerable, but incomplete, recovery of responsiveness to epinephrine occurred when the bathing medium containing PCMBS was replaced with PCMBS-free PSS (Fig. 3A). The extent of recovery was inversely related to the concentration of PCMBS initially tested. In sharp contrast, responsiveness to serotonin either was unchanged or deteriorated further when PCMBS-free PSS was returned to the muscle chamber (Fig. 3B).

PCMBS-induced inhibition of responsiveness to epinephrine was prevented when cysteine ($10^{-3} M$), an SH containing amino acid, was added to the bathing medium just before addition of $10^{-5} M$ PCMBS. Thus, the tension generated in response to epinephrine was 1463 ± 133 mg in 12 strips studied in the absence of PCMBS, and 1413 ± 107 mg ($P < 0.1$) after 120 min of contact with $10^{-5} M$ PCMBS and 10^{-3} cysteine.

Alpha-adrenergic blockade with $10^{-4} M$ phentolamine virtually abolished the contractile response to epinephrine. In eight strips tested, tension produced by epinephrine was 1348 ± 112 mg in the control setting and 108 ± 74 mg ($P < 0.001$) 30 min after incubation with phentolamine. Responses to serotonin were significantly ($P < 0.005$) reduced during alpha-blockade. Before blockade, responses to serotonin generated 1236 ± 107 mg of tension, but only 717 ± 44 mg was generated in the

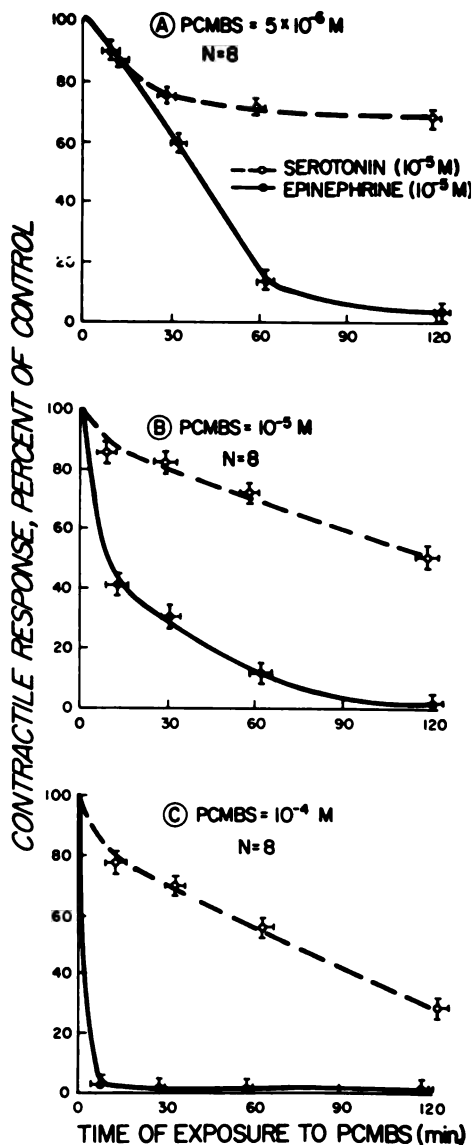
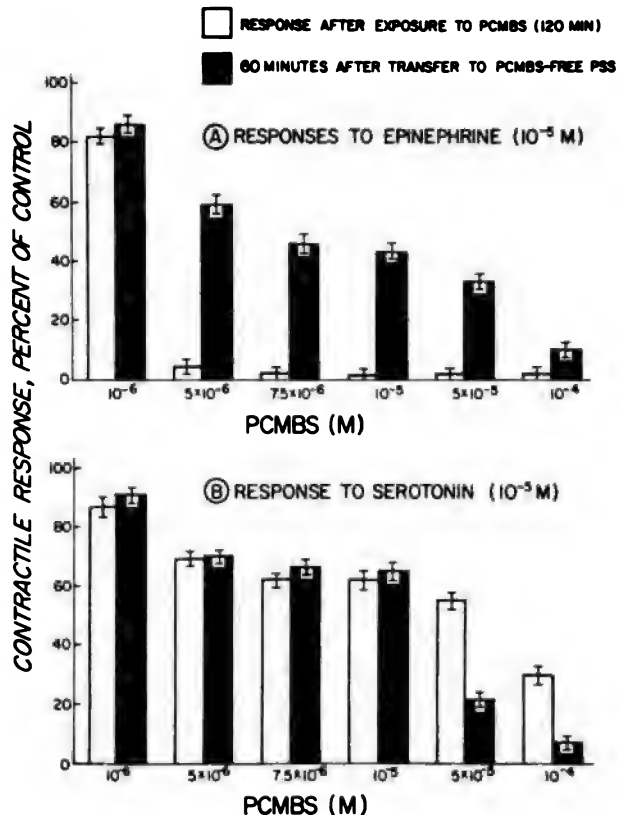


FIG. 2. Effects of different concentrations PCMBS on arterial responsiveness to epinephrine and serotonin. Different arterial strips were used for each concentration of PCMBS as shown in each panel. Small verticle bars represent 1 SE of the mean response (% of control), whereas horizontal bars represent 1 SE of the mean duration of contraction (min) with PCMBS. Inhibition of responses to epinephrine was always more rapid in onset and more pronounced than inhibition of responses to serotonin.

presence of phentolamine. Similar results were obtained with $5 \times 10^{-6} M$ phentolamine in eight different arterial preparations. In these strips, epinephrine increased tension to 1487 ± 136 mg before treatment



3. Responsiveness of arterial strips to epinephrine (panel A) and serotonin (panel B) 120 min after incubation with different concentrations of PCMBS (clear bars), and 60 min after replacing the medium with free PSS (dark bars). Small vertical bars represent ± 1 SE. Eight different arterial strips were tested at each concentration of PCMBS. Responsiveness to epinephrine recovered markedly after PCMBS was removed from the muscle chamber, but responsiveness to serotonin did not recover.

phenolamine, whereas only 196 ± 88 ($P < 0.001$) of tension were generated in the presence of phentolamine. Similarly, the tension generated in response to serotonin was 14 ± 100 mg before blockade, but 32 ± 44 mg ($P < 0.005$) of tension were generated after blockade with 5×10^{-6} M phentolamine.

Higher doses of isoproterenol (10^{-9} to 10^{-7} M) produced no change in tension (8 strips). However, higher doses produced small dependent increases in isometric tension. For example, 10^{-6} M isoproterenol increased tension to 57 ± 9 mg while tension increased to 136 ± 14 mg with 10^{-5} M isoproterenol. These contractile responses were completely abolished after alpha-adrenergic blockade with 5×10^{-6} M phentol-

Discussion. A prominent new finding in this study was that short periods of incubation (10–30 min) in the presence of low doses of PCMBS (10^{-6} to 10^{-5} M) markedly inhibited contractile responses to epinephrine in rat tail arterial smooth muscle. In contrast, longer periods of incubation (60–90 min) and higher doses of PCMBS (10^{-5} to 10^{-4} M) were required to markedly inhibit responses to serotonin (Figs. 1–3, Table I). These new findings suggest that SH groups may be important functional components of alpha-adrenergic receptors in arterial smooth muscle.

However, because epinephrine can stimulate both alpha and beta-adrenergic receptors, it is also conceivable that depression of responses to epinephrine with PCMBS is ascribable to potentiation of interactions

between epinephrine and beta-adrenergic receptors. This possibility does not appear likely because isoproterenol, though generally described as a beta-adrenergic stimulating agent (12), produced small increases in isometric tension. Furthermore, isoproterenol-induced contractions were blocked by phentolamine, an observation consistent with previous findings showing that isoproterenol can stimulate alpha-adrenergic receptors (13, 14). Accordingly, the inhibitory effects of PCMBS on contractile responses to epinephrine in tail arterial smooth muscle is likely due to interactions between PCMBS and SH groups associated with alpha-adrenergic receptors.

In contrast, responses to stimulation of serotonin receptors appear to be much less dependent on SH groups. An alternative hypothesis to account for the persistence of serotonin responses is that the accessibility of SH groups that might be associated with serotonin receptors is restricted so that their interaction with PCMBS is hindered.

Our interpretation of the data is based on studies in other laboratories which established that PCMBS is a potent and highly selective SH binding agent and that it is poorly permeant in a variety of cell types (1-6). In this context, our finding that cysteine, an amino acid with an SH group, protected arterial strips against PCMBS-induced inhibition of responsiveness to epinephrine suggests that the ability of PCMBS to bind SH groups is an important determinant of the efficacy of this compound to inhibit adrenergic responses. Furthermore, the rapidity of the onset of inhibition (Fig. 1) is consonant with the view that the inhibitory effect of PCMBS is mediated at sites close to the exterior of the smooth muscle cell, perhaps at the level of the plasma membrane. This view is also supported by the observation that marked and rapid recovery of responsiveness to epinephrine occurred when the bathing medium was replaced with PCMBS-free PSS (Fig. 3A).

Surprisingly, recovery of responsiveness to serotonin did not occur when PCMBS was washed from the muscle chamber (Fig. 3B). Thus, at each dose of PCMBS tested, responses to epinephrine were consistently more depressed than were responses to serotonin, but the fractional recovery of re-

sponsiveness was always greater with epinephrine. The mechanistic basis for this interesting observation is unknown. Nevertheless, it is noteworthy that recovery of responsiveness for either epinephrine or serotonin was always incomplete, suggesting that a fraction of the PCMBS initially included in the incubation medium was tightly, perhaps irreversibly, bound to the smooth muscle strips.

The present findings that alpha-adrenergic blockade with phentolamine abolished responses to epinephrine and significantly attenuated responses to serotonin are consistent with results obtained by other investigators studying human umbilical arteries and veins (15), rabbit portal vein (16), and canine saphenous, jugular, and mesenteric veins (17). These results suggest that vascular smooth muscle receptors for serotonin and epinephrine may be structurally related. It is tempting to speculate that such structural relationships could account for, or contribute to, the partial inhibition of serotonin-induced contractions by PCMBS (Fig. 1, Table I). Further studies are required to test this hypothesis.

We recognize that PCMBS may influence arterial responsiveness to agonists by mechanisms other than those involving interactions with SH groups of receptor moieties. However, our findings showing that the inhibitory effects of the compound were selective, time and dose-dependent, rapid in onset, partially reversible, and preventable by cysteine, strongly suggest that PCMBS interacts with SH groups present in arterial smooth muscle receptors for vasoactive stimulants.

Nevertheless, it is important to emphasize that different compounds which interfere with SH groups may produce different effects on the responsiveness of arterial smooth muscle to vasoactive stimulants. For example, Needleman *et al.* (18) reported that alkylation of SH groups with ethacrynic acid did not alter contractile responses elicited with norepinephrine in rabbit aortic strips. In contrast, Fleisch *et al.* (19) found that alkylation with *N*-ethylmaleimide significantly reduced responses to norepinephrine in similar preparations from rabbit aorta. The reasons for these disparate findings are unclear but may be related to differ-

between molecular size and charge of the alkylating agents (1, 2). Our results with rat tail arterial smooth muscle are in accord with those reported by Fleisch *et al.* (19) in that high concentrations of *N*-ethylmaleimide (10^{-4} M in aorta), like high concentrations of α -methyl-5-hydroxytryptamine (rat tail artery), inhibited responses to serotonin.

Summary. The purpose of this study was to gain insight into the role of sulfhydryl groups in arterial receptors for vasoactive agents. We studied the effects of *p*-mercuriphenyl sulfonate (PCMBS, 10^{-4} M), a potent and specific SH reagent, on contractile responses produced by 10^{-5} M epinephrine and 10^{-5} M serotonin in helical strips of arterial smooth muscle prepared from tail arteries of the rat. Responses to epinephrine were rapidly, and reversibly inhibited with low concentrations of PCMBS. In contrast, responses to serotonin were slowly inhibited and high concentrations of PCMBS were required.

The inhibitory effect of PCMBS on epinephrine-induced contractions was prevented by 10^{-3} M cysteine. Adrenergic blockade with phentolamine (10^{-4} or 5×10^{-6} M) virtually abolished responses to epinephrine and significantly decreased responses to serotonin. These findings are consistent with the view that arterial receptors for contractile responses to epinephrine and serotonin may be structurally related. The data suggest that SH groups are important functional components of adrenergic receptors, but they are of lesser importance for responsiveness of serotonin receptors.

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The Activity of a Thiadiazole on *Mycobacterium leprae* (39475)

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A new compound, 2-amino-5-(1-methyl-5-nitro-2-imidazolyl)-1,3,4-thiadiazole (CL 64,855), was reported (1, 2) to exhibit a broad spectrum of antibacterial and antiprotozoal effects upon oral administration to chicks and rodents. The compound was not found active against *Mycobacterium tuberculosis* (H. Macdonald, personal communication). In a series of experiments, we have found that CL 64,855 is active against *M. leprae*. The drug produced either bacterial killing or prolonged bacteriostasis, an attribute of very few of the large number of compounds already studied (3).

Methods. CL 64,855 was incorporated into mouse meal (Wayne Lab Blox, Allied Mills, Inc., Chicago, Illinois) by the addition of appropriate volumes of a solution of the compound in 95% ethanol in a liquid-solid twin-shell blender (Patterson-Kelly Co., East Stroudsburg, Pennsylvania). The drug-containing diets were placed in powder feeders in the mouse cages and made available to the mice *ad libitum*.

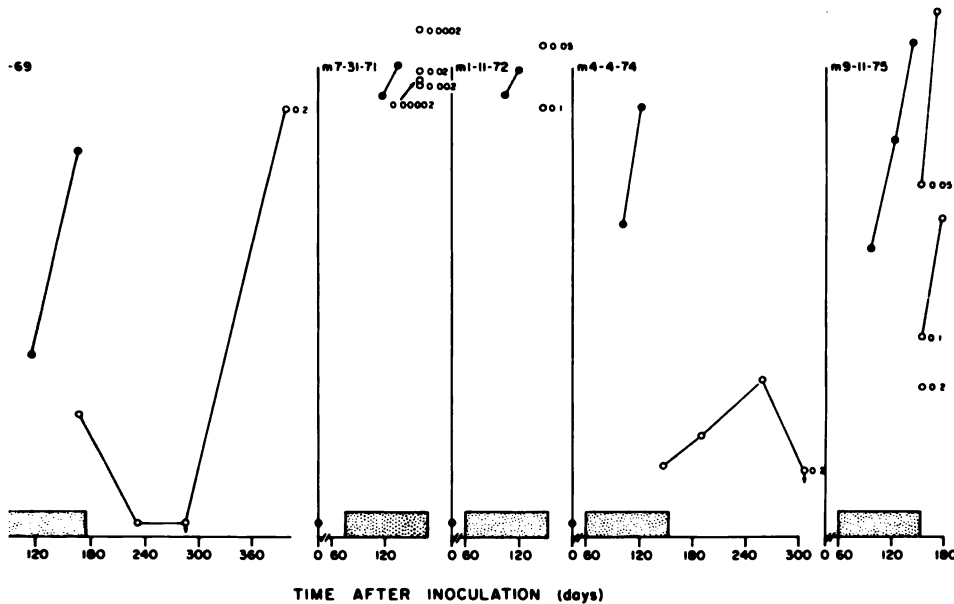
Locally-bred male BALB/c mice were inoculated in both hind foot pads with $10^{3.7}$ *M. leprae* of the strain used for virtually all of the drug studies carried out in this laboratory. Drug-containing diets were administered for periods of approximately 90 days, beginning 60 to 75 days after inoculation, when the organisms were in logarithmic multiplication. *M. leprae* were harvested and enumerated at intervals from both untreated control mice and treated animals by published methods (4, 5).

Results. The results of five experiments in which CL 64,855 was administered to *M. leprae*-infected mice are presented in Fig. 1. In the first experiment (m 9-3-69), a harvest of *M. leprae* from the foot pads of untreated control mice performed just before drug administration was terminated yielded an average of $10^{5.7}$ organisms per foot pad. A harvest performed on the same day from the foot pads of mice to which CL 64,855 had

been administered in a dosage in the 0.2 g% yielded $10^{4.3}$ acid fast bacilli per foot pad. Two subsequent harvests of treated mice, performed at interval termination of the treatment, yielded numbers of AFB no larger than that inoculated. Finally, a harvest performed 398 days after inoculation and 228 days after drug administration had been stopped yielded $10^{4.3}$ AFB per foot pad. The organisms apparently have multiplied at the same rate as untreated mice during the period between 228 and 398 days after inoculation.

In the second experiment (m 7-3-72) dosages of CL 64,855 ranging from 0 to 0.02 g% appear to have been without effect on the multiplication of *M. leprae*. The results of the third experiment (m 7-2-72) suggest that the drug had no effect when administered in a concentration of 0.05 g% whereas it may have exerted a modest effect on multiplication of *M. leprae* when administered in a concentration of 0.1 g%. Because of an insufficient supply of the drug and because there was no reason to expect a steep dose-response curve implied by these results, the concentration of CL 64,855 previously found effective at 0.2 g%—was not retested concurrent with the testing of the lower concentrations.

In order to be certain that the effect on multiplication of *M. leprae* observed in the first experiment could be confidently attributed to the action of the drug, 0.2 g% CL 64,855 was administered in a fourth experiment (m 4-4-74). Profound inhibition of growth of the organisms was again observed. Finally, in the fifth experiment (m 11-75), the titration of the drug was repeated, on this occasion concurrent with the administration of 0.2 g% CL 64,855. As shown in the right-hand panel of Fig. 1, the drug exerted an effect on multiplication of *M. leprae* when administered in dosages of 0.1 and 0.2 g%; in addition, the administration of 0.05 g% of the drug appears to



Log₁₀ number AFB per foot pad as a function of time after inoculation. Mice were inoculated with *rae* in both hind foot pads (point on each ordinate). AFB were harvested at the intervals shown from issues of four foot pads: closed circles—control mice; open circles—mice treated with CL 64,855 for the *wn* by the shaded bars along the abscissa. The concentrations in g% of drug administered to the mice by the numbers appearing next to the points representing harvests from treated mice. Those points with extending arrows represent harvests from which no AFB were recovered; the results were calculated as *ism* had been counted.

a modest effect. The administration of 0.05 nor 0.1 g% CL 64,855 delayed the resumption of multiplication of *M. leprae*. Unfortunately, the automatic watering system in the house caused the loss of so many mice in this experiment that none of the mice treated with 0.2 g% of the drug were available for later harvest.

Conclusion. The purpose of this study was to evaluate a new compound for activity against *M. leprae* in the mouse foot pad and to attempt to characterize the antibacterial effect. The results demonstrated that CL 64,855, a thiadiazole derivative, has a broad spectrum of antimicrobial activity but no activity against *M. tuberculosis*. It has a profound effect on the multiplication of *M. leprae*.

The drug was without effect when administered in the mouse chow in concentrations of 0.05 g%. In a dosage of 0.05 g% the drug was without effect on one mouse and produced modest inhibition of multiplication of the organisms on a second mouse. Administration of CL 64,855 in a concen-

tration of 0.1 g% was modestly effective on one occasion, and demonstrated a definite effect in another experiment. When administered in a dosage of 0.2 g%, the drug exerted a profound effect on multiplication of *M. leprae* in three experiments.

After multiplication of *M. leprae* in the mouse foot pad has been interrupted by the administration of an effective drug and drug administration is subsequently terminated, the organisms resume multiplication in one of three ways (3). Immediate resumption is characteristic of the action of a drug that has exerted only a bacteriostatic effect, whereas the failure of multiplication may be taken as evidence of a bactericidal effect that has resulted in eradication of the *M. leprae* infection. More difficult to interpret is the situation in which the organisms resume multiplication only after a delay longer than can be attributed to the continued presence of the drug in an effective concentration. This situation is exemplified by the results of administration of CL 64,855 in the largest concentration studied. The delay before resumption of multiplication may be attrib-

uted to a prolonged bacteriostatic effect of the drug; the administration of dapsone frequently produces such a delay (6, 7). Alternatively, the delay may be attributed to a bactericidal effect of the drug that falls short of eradication of the infection. In this latter instance, the delay occurs because multiplication resumes from only the surviving fraction of the population of *M. leprae*, and, although the surviving organisms may resume multiplication immediately upon withdrawal of the drug, the first generations result in numbers of AFB that are too small to be detected. This interpretation appears to fit best the data shown in Figure 1. Particularly in experiment m 4-4-74, the suggestion that CL 64,855 has exerted a bactericidal effect is supported by the failure to find any organisms in the foot pad tissues of the mice sacrificed after 305 days, whereas small numbers of AFB had been enumerated in earlier harvests. It appears likely that the infection had indeed been eradicated in the foot pads of some of the mice.

In a survey of drugs studied for activity against *M. leprae* in the mouse foot pad infection, Shepard pointed out (3) the importance of those few agents capable of producing a delay of resumption of multiplication. This demonstration that CL 64,855 in sufficient dosage produces such a delay suggests that this drug or analogous compounds may potentially have value in the treatment of patients with leprosy. And the large dosages well tolerated by the mice suggest that the compound is relatively nontoxic. Unfortunately, the drug has been withdrawn from human trial because of its carcinogenicity on

long-term administration to rats (H. Donald, personal communication). Perhaps some analog will prove to be both effective against *M. leprae* and free of carcinogenic effects.

Summary. A new broad-spectrum antimicrobial, 2-amino-5-(1-methyl-5-nitroimidazolyl)-1,3,4-thiadiazole, reported effective against *Mycobacterium tuberculosis* inhibited multiplication of *M. leprae* in mouse foot pad when administered to the mice. The dose-response curve was steep: 0.2 g% of the drug exhibited considerable activity, whereas 0.05 g% was modestly active in one experiment and inactive in another. This drug appears to be one of the few that is bactericidal for *M. leprae*.

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Further Evidence for the Existence of Angiotensinogen Stimulating Activity (ASA) after Nephrectomy¹ (39476)

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preincubation with plasma from nephrectomized animals liver slices from rats released larger amounts of angiotensinogen than slices which had been incubated with normal plasma (1). This observation together with results of liver biopsies (2) led to the suggestion that the decrease in circulating angiotensinogen seen after nephrectomy is due mostly to an increase in synthesis elicited by an angiotensin-stimulating factor. Some possibility exists, however, that the larger amounts of angiotensinogen present in the incubation medium might represent release of material from the liver during the preincubation period with angiotensinogen rich plasma. Preliminary experiments in which liver slices were incubated with semipurified angiotensinogen were inconclusive (1). The present paper demonstrates that stimulation of angiotensinogen synthesis can be observed with a portion of plasma virtually free of angiotensinogen. It also includes further studies on the nature of the stimulus.

Materials and Methods. *DEAE Cellulose chromatography.* The method of Cook and Lee (3) for purification of angiotensinogen was used with the hope that it might permit separation of the angiotensinogen from angiotensinogen stimulating activity (ASA). Plasma from 48 hr nephrectomized rats was purified by ammonium sulfate precipitation at 40% saturation and then subjected to ion exchange chromatography on DEAE cellulose. The separation was carried out with a linearly increasing concentration of sodium chloride in 0.05 M Tris-HCl buffer pH 7.5 at a rate

of 60 ml per hour, and was monitored by UV absorption at 280 nm. The eluate was collected in fractions of 12 ml in 136 tubes. Protein composition of the fractions was determined by polyacrylamide gel electrophoresis (4). Angiotensinogen and ASA were determined by bioassay. Angiotensinogen was determined directly on 1 ml of fluid. For ASA determination, the remaining content from each tube was concentrated by evaporation to half its volume and dialyzed against Robinson solution (5). Then aliquots of 3 ml were preincubated with normal liver slices for measurement of ASA.

Starch powder electrophoresis. The electrophoretic method of Kunkel and Slater (6) was carried out on 5 ml portions of serum from nephrectomized rats and on equivalent amounts of the active fraction obtained by precipitation with ammonium sulfate. In the latter instance, the precipitate was dissolved in water and dialyzed against 0.9% NaCl solution. Serum required no prior treatment. At the end of the electrophoresis, the protein pattern was identified from a paper strip which had been applied on the starch and stained for proteins. From the pattern obtained, the starch block was cut into segments which were suspended in saline. The suspension was centrifuged and the supernatant was concentrated by evaporation to the original serum volume. After dialysis against Robinson solution, the eluted material was analyzed for content of angiotensinogen and ASA, nitrogen content by Kjeldahl digestion and nesslerization, and protein composition by cellulose acetate electrophoresis.

Chromatography of Sephadex. The ASA-active portions of the eluate obtained by chromatography on DEAE cellulose were combined and concentrated by evaporation from 10 ml to 1.5 ml. This amount was applied on a 30 ml column of Sephadex G-

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200 that had been equilibrated with Robinson solution. Elution was carried out with Robinson solution at a rate of 3 ml/hr, and monitored by uv absorbance at 280 nm. Fractions (1 ml) of the effluent solution were collected for a total of 30 tubes. All proteins were contained in tubes 10 to 30. These 1 ml fractions were combined to give seven subfractions with volumes ranging from 2 to 4 ml in approximate proportion to the breadth of protein boundaries evident from electrophoresis. The subfractions were made up to a volume of 4 ml and were preincubated with normal liver slices for determination of ASA.

Biological methods. Female Sprague-Dawley rats weighing approx 200 g were fed a commercial chow and given tap water to drink. Normal and 48 hr nephrectomized animals were used to provide plasma, serum, or liver slices. Plasma was obtained by withdrawing blood during amobarbital anesthesia (9 mg/100 g) in a syringe moistened with 0.3 M disodium EDTA. Plasma and serum were used fresh or after freezing.

Determination of ASA. After laparotomy during amobarbital anesthesia (9 mg/100 g) a PE 60 plastic cannula was inserted into the portal vein. Aorta and vena cava were sectioned above the diaphragm and the liver was flushed with 30 ml of saline followed by 30 ml of cold Robinson solution. Slices 0.5 mm thick were cut, washed three times in 15 ml of Robinson solution, and drained on filter paper. Portions weighing 200 mg were placed in Warburg flasks containing the material to be tested, dissolved in 3 ml of Robinson solution. In control experiments the medium consisted only of 3 ml of Robinson solution or of normal plasma or serum. The flasks were then placed in a Gibson differential respirometer at 37°C, shaken for 1 hr at a frequency of 120 times per minute and ventilated with a mixture of 95% O₂ and 5% CO₂. Following this preincubation the medium was discarded, the slices were washed twice with 50 ml of Robinson solution, suspended in 3 ml of fresh Robinson solution and incubated for a period of 4 hr under conditions identical to those described above. At the end of the incubation, the content of each flask was centrifuged and the supernatant fluid used for determi-

nation of angiotensinogen. Rates of angiotensinogen released and presumably formed during incubation are expressed in nanograms of angiotensin per gram of liver per hour of incubation (ng angiotensin/g/hr). Rates exceeding the control value indicate presence of ASA in the preincubating medium.

Determination of angiotensinogen. Angiotensinogen concentration was estimated from the amount of angiotensin generated following incubation of samples with an excess of renin (7). Angiotensin was bioassayed in pentolinium treated rats (5 mg of the tartrate/100 g) using angiotensin II (Hypertensin, Ciba) as standard. Angiotensinogen concentration is expressed in nanograms of angiotensin per milliliter (ng angiotensin/ml). Values are means \pm SD.

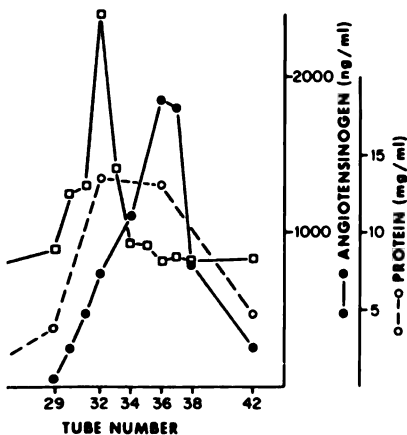
Effect of Renin on ASA. The purpose of the experiment was to explore the possibility of a cause-effect relationship between the low renin and the high ASA associated with nephrectomy. Plasma (3 ml) from 15 hr nephrectomized rats was incubated for 15 min in the presence of 25 Goldblatt units of semipurified renin contained in 0.1 ml of saline (2). At the end of the incubation it was cooled in an ice bath and dialyzed against Robinson solution for 24 hr to remove angiotensin and other products which may have resulted from the proteolytic action of renin and other peptidases. It was then assayed for angiotensinogen concentration ASA. Control tubes contained plasma incubated with renin which had been inactivated by boiling.

Results. Validation of the procedure for determination of ASA is supported by the following results. Without preincubation, normal liver slices incubated for 4 hr in Robinson solution released angiotensinogen at the rate of 9.3 ± 0.92 ng angiotensin/g/hr. Similar values were obtained with preincubation for 1 hr in Robinson solution or normal plasma, averaging respectively 8.7 ± 1.04 and 9.7 ± 1.47 ng angiotensin/g/hr. However, rates were markedly increased by preincubation in plasma from nephrectomized animals to average 21.0 ± 2.72 with 5 hr nephrectomized plasma and 41.94 ± 4.66 with 15 hr nephrectomized plasma.

DEAE cellulose chromatography. Chro-

hs of two batches of serum from omized animals gave similar reth the first batch angiotensinogen d in tubes 30 to 40 with a peak g to 1825 ng of angiotensin per ml of angiotensin per mg protein) in (Fig. 1). On the other hand, ASA nt in tubes 30 to 33 with a peak of angiotensin/g/hr in tube 32. The taining the largest amounts of anogen did not show significant lues varied between 10.2 and 11.7 tensin/g/hr as compared with cones between 9.7 and 11. With the atch, both peaks were slightly diso the left. Angiotensinogen was i tubes 24 to 34 with a maximum of ml in tube 30, while ASA was preses 23 to 27 with a peak of 33.7 ng in/g/hr in tube 26. Again, the o to 31) which contained the largest of angiotensinogen did not show t ASA. The peak of angiotensinoluted with a chloride concentration M and that of ASA with a concen: 0.078 M .

phoresis of the various fractions by chromatography (Fig. 2) hat the fractions rich in ASA and inogen contained numerous comsome of which varied in apparent n to ASA and angiotensinogen itions. However, we could not asy single component with each of iological activities.



Separation of angiotensinogen from ASA elution from DEAE-cellulose.

Starch powder electrophoresis. Following electrophoresis of serum from nephrectomized animals, significant amounts of ASA were found only in the γ_2 -globulin fraction (Table I). Rates of angiotensinogen formation in the other fractions did not significantly differ from the control value. The concentration of ASA in the γ_2 -globulin fraction is high both in absolute value and in terms of protein concentration. In contrast, angiotensinogen activity was found in the fraction with albumin- α_1 -globulin mobility. Results obtained by electrophoresis of the material precipitated at 40% ammonium sulfate saturation were not informative because the activity was more or less evenly distributed between all three of the major protein zones: albumin, $\alpha + \beta$ globulins, and $\gamma_1 + \gamma_2$ globulins.

Chromatography on G 200 Sephadex. The active fractions rich in ASA derived from DEAE cellulose chromatography were separated according to molecular size of its constituents by chromatography on Sephadex G 200 (Fig. 3). Assay of effluent for ASA showed that the bulk of activity was eluted in the fractions containing proteins of molecular weights around 60,000.

Effects of renin on ASA. Results from two experimental series using the same pool of plasma were similar, and differences between values were less than 10%. As summarized in Table II, they show that incubation with active renin (tubes 4 and 5) caused the destruction of both angiotensinogen and ASA, while addition of boiled renin had no effect on either angiotensinogen or ASA. Pretreating plasma by dialysis (tubes 1 and 2) lowered ASA but not angiotensinogen.

Discussion. The present experiments confirm our previous observation (1) that after nephrectomy the plasma acquires the property of stimulating angiotensinogen formation, hence that this property is likely related to the spectacular rise in circulating angiotensinogen. They also demonstrate that ASA is amenable to purification by electrophoresis and chromatography.

It has been shown that the liver does not store any appreciable amounts of angiotensinogen and that whatever is present in a liver perfusate (2) or in the incubating medium of liver slices (7) represents newly synthesized material. Nevertheless, since most

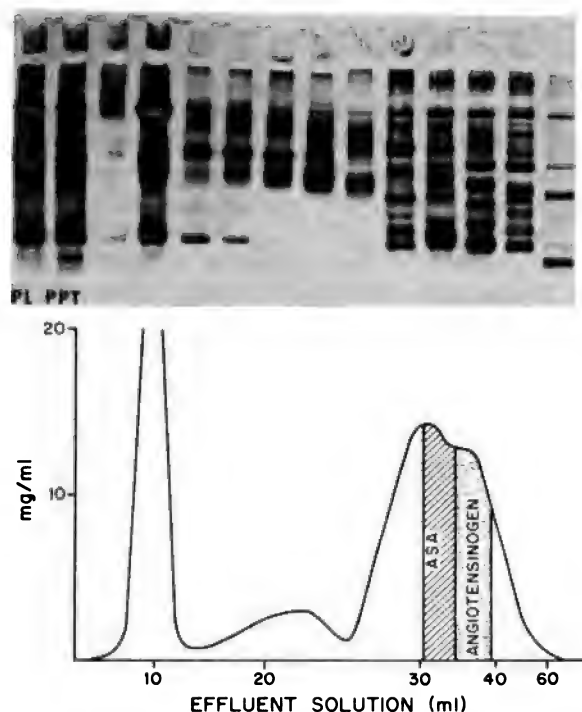


FIG. 2. DEAE chromatography of ammonium sulfate precipitate containing ASA and angiotensinogen. The elution profiles of the various components in the starting material are portrayed by the superimposed polyamide-gel electrophoresis patterns. The abscissa of the chromatogram was drawn in nonlinear scale to align with the electrophoretic patterns. As judged by resemblance to patterns obtained with both plasma and the precipitate (PPT), tubes 9-11 contained protein applied in excess of binding capacity of the DEAE. Absence of ASA and angiotensinogen in this fraction indicated they were fully retained. The crosshatched and stippled areas depict location of peak activities of ASA and angiotensinogen in the eluate.

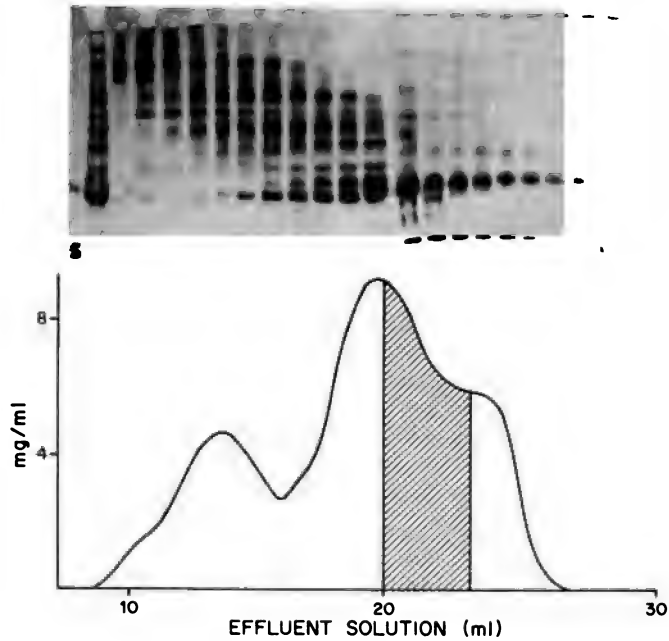
TABLE I. EFFECTS OF FRACTIONS FROM STARCH-POWDER ELECTROPHORESIS ON ANGIOTENSINOGEN FORMATION.

Fractions	Protein ^a concentration (mg/ml)	Angiotensinogen ^a concentration (ng angiotensin/ml)	Rates of angiotensinogen formation (ng angiotensin/g/hr)
Albumin + α -globulin	10.50	200	9.5
α 2-globulin	1.32	34	9.0
β -globulin	2.45	30	9.2
γ 1-globulin	0.38	32	9.7
γ 2-globulin	0.10	32	15.7
Original serum	64	2850	43.2
Control	—	—	9.7

^a Measurements made on preincubation medium.

of the active materials which were previously tested for ASA contained large amounts of angiotensinogen and since the amounts of angiotensinogen released by

slices are relatively small, one could discard the possibility that some angiotensinogen might be absorbed during the incubation to be later released during the incubation. Another possibility, however remote, is that angiotensinogen itself in an altered form of it may act as a stimulus. The first possibility may be discarded on the following evidence. First, plasma contained more ASA than serum although both were obtained from the same animals and had the same angiotensinogen concentration. Secondly, on electrophoresis of serum, ASA was present in the γ 2-globulin fraction whereas angiotensinogen moved with albumin- α 1-globulin. Thus, ASA and angiotensinogen have opposite positions in the electrophoretic pattern (8). Lastly, chromatography on DEAE cellulose revealed that the ASA and angiotensinogen were separated separately, thus eliminating the possi-



3. Gel filtration chromatography on G 200 Sephadex of DEAE fractions containing ASA. The cross-hatch area demarcates portion of the effluent solution with peak ASA. The accompanying polyacrylamide-gel electrophoresis patterns showed that this portion contained small sized proteins such as albumin.

TABLE II. EFFECTS OF RENIN ON ASA AND ANGIOTENSINOGEN IN PLASMA FROM NEPHRECTOMIZED ANIMALS.

	1	2	3	4	5
renin	0	0	0	+	+
renin	+	+	+	0	0
tion	0	0	+	+	+
s	0	+	+	0	+
ensinogen (ng angiotensin/ml)	3600	3500	3300	N.M. ^b	N.M. ^b
of angiotensinogen formation	39.2	26.1	25.5	6.9	7.6
ngiotensin/g/hr)					

^a 1 tubes contained 3 ml of plasma from 15 hr-nephrectomized rats.
^b not measurable.

angiotensinogen was responsible for the observed differences in physical properties dispel the previous suggestion that ASA might somehow be due to angiotensinogen itself, the possibility of an altered form of angiotensinogen possessing warrants consideration. If alteration is attributed, the change would have to be related to nephrectomy by virtue of appearance of ASA in nephrectomized animals, (ii) unrelated to the assay system or concentrations of angiotensinogen incubated with liver slices because of lack of effect in purified angiotensinogen, and (iii) unrelated to changes occurring in

plasma because ASA did not increase on storage. It had also been noted before that incubation with a semipurified renin preparation destroyed ASA in plasma. Since these considerations largely eliminate possibility of change occurring in plasma and liver, the principal sources of angiotensinogen, we presently view the conjectured alteration as only a remote possibility.

Except that it is very likely a protein, we have little information on the nature of the substance responsible for ASA. Electrophoresis and ultracentrifugation of serum suggest a high molecular weight protein behaving like a γ -globulin. On the other hand, gel filtration of material that had been purified

by ammonium sulfate precipitation and DEAE chromatography indicates a smaller molecule behaving like albumin. This contradiction may, however, be only apparent if we postulate that the substance has either high density, possibly corresponding to a glycoprotein, or exists as a complex with other protein, seemingly γ -globulin in serum. The latter postulate is based on the following observation. Electrophoresis of concentrates of ASA prepared by ammonium sulfate precipitation and dialysis gave results that differed from electrophoresis of serum on each of two trials in which serum and concentrates were compared. The observed association of ASA with all of the major protein fractions separated by electrophoresis subsequent to ammonium sulfate precipitation suggests that binding affinities were altered by the precipitation. The apparent alteration of binding affinities by the precipitation procedure do not invalidate distinctions made in properties of ASA and angiotensinogen because they were separable from each other both before and after the precipitation, the electrophoretic separation being performed before, and the DEAE chromatography after.

The specific activity of ASA purified by the electrophoresis of serum far exceeded that achieved by all the other attempted purification methods combined. The high efficiency of this separation method is attributable to the migration of ASA with the γ 2-globulins which comprise only a small percentage of the serum proteins. The active subfractions from DEAE chromatography and gel filtration subsequent to ammonium sulfate treatment contained numerous electrophoretic components, albumin being a major one. Since large losses in activity did not occur, the low specific activity achieved by the latter methods was undoubtedly due to the high content of albumin in the subfractions. From observations made thus far, it would appear that a very high degree of purification might be achieved by electrophoresis followed by ammonium sulfate precipitation and gel filtration, but this possibility remains to be tested pending availability of material.

The substance responsible for ASA is not

unique. Estrogens (2), cortisol (7), and angiotensin (9), are also known to directly stimulate angiotensinogen formation. The report (7) that cortisol injected into normal rats mimicked the rate of angiotensinogen formation elicited by nephrectomy is not in accord with results from experiments in which cortisol was added to the medium perfusing a normal liver: Rates were increased but not as much as after nephrectomy (10). It is very likely that the greater rates found after cortisol injections in non-nephrectomized animals were due to the stimulating activity of angiotensin superimposed on that of the steroid. Indeed any procedure which would result in a stimulation of the pituitary adrenal system should be considered of questionable value for the detection of ASA, especially when a rise in plasma angiotensinogen is used as criterion (11).

ASA is undoubtedly an extrarenal factor, because of its appearance in blood after nephrectomy. It is not clear whether formation of ASA might be specifically associated with the uremic state or might possibly represent the accumulation of a physiologically formed substance which under normal conditions is excreted or destroyed by the kidney. The present observations on the inactivating effect of renin could provide an explanation for its destruction when renin levels are high or normal and its accumulation when renin secretion ceases. However, this point will require study of kinetics of the inactivation.

The evidence that many factors including the one appearing after nephrectomy have the property of stimulating angiotensinogen synthesis and that some of these factors are able to affect directly both angiotensinogen formation and renin secretion emphasize the complexity of angiotensinogen regulation as well as the complexity of the interrelationships existing between the various components of the renal-pressor system (10). The renal-pressor system is known to be well integrated and to possess multiple compensatory pathways for maintenance of vascular turgor. The extent to which ASA may participate in autoregulation cannot be predicted at present because of the unex-

possibility that its appearance in urine might specifically depend on nephrec-

tomy. After nephrectomy, plasma renin acquires the property of stimulating angiotensin (A) synthesis in liver slices of normal rats. The possibility that this renin-stimulating activity (ASA) is an artifact arising from exposure of the liver to the high levels of A in the test system has been examined. It has now been found that ASA and A can be separated by electrophoresis on starch powder and by gradient elution from DEAE cellulose. The identification of ASA as a distinct component of the renal-pressor system raises several aspects for investigation of control mechanisms within the system.

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Enumeration of Specific Antibody-Forming Cells of the Mouse Spleen after Stimulation with Protein Antigens¹ (39477)

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In the experiments to be reported here, cell counts trace the rise and fall of the population of antibody-forming cells in the red and white pulp of the spleen of the mouse during both initial and recall responses to the injection of antigen. Human IgG (HGG), heated β -galactosidase (BGz) from *E. coli*, and keyhole limpet hemocyanin (KLH) were used. In addition, the effects of a wide variety of doses on the distribution and number of cells making a specific response were studied for the cases of BGz and KLH.

Over the years White and his colleagues have made a systematic study of the localization of antigen and the appearance of antibody in the chicken spleen, using human serum albumin (HSA) as the antigen (1-3). The first antibody-producing cells occurred in the red pulp by the 30th hr, and only later did antibody appear in the germinal centers. They found traces of antibody, then increasing amounts of antigen, then more antibody in the germinal centers. Our data extend those of White and his colleagues by enumerating the cells and indicate the similarity of antibody-location in the mouse and the chicken. Urbain-Vansanten and her colleagues (4, 5) have counted the antibody-containing and immunoglobulin-containing cells in suspensions of rabbit spleen after antigen stimulation with several antigens; all were injected several times to achieve primary stimulation, without adjuvant. They found about five cells/thousand to contain antibody, and about 15/thousand to contain immunoglobulin not identifiable as specific antibody, but nevertheless in re-

sponse to the antigenic stimulus in some perhaps indirect way. The work presented here confirms Urbain-Vansanten *et al.* and adds information about the location of the cells in the spleen.

Materials and methods. Antigens. Mouse γ -globulin was prepared from the serum of A.I. mice (Kyoto University Animal Center) by ammonium sulfate fractionation and DEAE cellulose chromatography (6). Purified β -galactosidase of *Escherichia coli* (360 units/mg BGz) was obtained from Worthington Biochemical Corp. (Freehold, New Jersey). The keyhole limpet hemocyanin (immunologically pure, KLH) was obtained from Calbiochem (Los Angeles, California). These were used as antigen without further purification.

Preparation of antisera and conjugates for immunofluorescence studies. Anti-BGz and anti-KLH sera were prepared in male albino rabbits and analyzed by double diffusion in agar. Neither cross-reacted with any antigen in mouse serum. Anti-MGG was tested against mouse serum after electrophoresis; it reacted with three antigens, all of which were in the region of γ -globulins and no others.

Globulin fractions of these antisera were conjugated with fluorescein isothiocyanate (7) as was HGG. Another fraction of the anti-BGz antibody was coupled with rhodamine isothiocyanate (8). These conjugates were fractionated on DEAE cellulose and concentrated by dialysis against polyethylene glycol (20,000 daltons).

Animals and immunization. Both sexes of inbred mice (body weight 18-25 g) were used in this study. Inbred C₃H mice were obtained from Kyoto University Animal Center (Kyoto) and Jackson Laboratories (Bar Harbor, Maine). A/Jax mice were obtained from Jackson Labs. Experimental animals in one series were given primary injections of 5 mg of soluble HGG into a tail vein. In other series heated BGz or KLH in

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doses was administered intraperitoneally for the secondary response to BGz, a heat-killed BGz was given ip in complete adjuvant (Difco Labs) as a first dose, followed by another dose without adjuvant 4 weeks later.

Groups of two to three mice were killed at days specified after secondary stimulation. Dose response curves for these two experiments were also carried out using two or three mice per dose.

Preparation of sections. Spleens were removed and cut lengthwise into three strips with a sharp razor. The middle strip of each spleen was placed quickly in cold 95% ethanol, held for 18 hr at 4°C, and embedded in paraffin according to the procedure of Boulikas-Marie (9). Longitudinal sections (5 μ m thick) were cut, deparaffinized in xylene, and hydrated through successive alcohol baths.

Staining with fluorescein-labeled conjugates for fluorescence microscopy. For the staining of anti-HGG antibody sections exposed to labeled antigen, i.e., fluorescein-labeled HGG that had been adsorbed twice with mouse liver or spleen extract.

The exposure continued overnight in the dark with dilute conjugate (about 0.25 μ g/ml). In the case of BGz-injected mice, sections were first exposed to rhodamine-labeled anti-BGz to detect any antigen remaining from the injection; none was detected. They were then stained by the "sandwich" technique for anti-BGz (0.1 mg/ml PBS with 10% fetal calf serum for 2 hr) and then exposed to fluorescein-labeled anti-BGz for 1 hr).

For anti-KLH antibody, similar procedures were used without preliminary exposure to labeled anti-KLH. For MGG, sections were stained with fluorescein-labeled anti-MGG for 2 hr or overnight. All incubations were carried out in a moist chamber at room temperature.

Stained slides were washed with PBS, mounted in glycerol containing 10% PBS, and examined under a Zeiss fluorescence microscope equipped with an HBO 200 high pressure mercury vapor lamp (Osram, Munich, Germany) using a Corning 44 exciter filter, a dark field condenser, and a Zeiss 41 barrier filter.

Specific stained cells were counted

throughout the area of a section under a fluorescence microscope using a 12.5 \times eyepiece ocular and a 40 \times objective. The approximate size of the section was estimated with the aid of a piece of graph paper ruled in millimeters. At least three good sections were examined per spleen.⁵

Results. The spleen consists of two main tissues, the white pulp, which sheathes the arteries and their small branches as they enter and ramify, and the red pulp, into which they empty blood. In addition, it is possible to identify a marginal zone, a broad ill-defined junctional tissue lying between the white and the red pulp. It receives much of the blood and is the initial area of concentration of antigen, and of the differentiation of lymphoid cells and macrophages. In the white pulp, round collections of lymphoid cells develop, the lymphoid follicles, and in their centers sometimes germinal centers. Around the germinal center is a cuff of small lymphocytes called the "mantle layer." (10).

The primary response. The first cells containing detectable antibody against HGG were found 8 hr after the injection of antigen in the red pulp; They were large mononuclear cells with a large nucleus and a thin rim of cytoplasm. Some of them showed fluorescence in only a portion of the cytoplasm. They occurred singly, their number slowly increasing during the first 4 days. Between the second and the fourth days the number of cells in the red pulp increased about twice, but now the gains began to shift to the white pulp; here during the same period the number increased 20 times. By the eighth day there were four times as many cells in the white pulp as in the red. This represented the peak; thereafter, the reaction gradually subsided (Fig. 1).

⁵ For convenience, we have calculated roughly how many spleen cells the unit area of the spleen sections corresponds to. Assuming that the area of a spleen section is occupied by cells with a diameter averaging 10 μ m, we estimate that 100 mm² of surface area corresponds to about $1-1.3 \times 10^6$ spleen cells. Abbreviations used in this paper: HGG, human gamma globulin; BGz β -galactosidase of *E. coli*; KLH, Keyhole limpet hemocyanin; MGG, mouse gamma globulin; DEAE, diethylaminoethyl cellulose; F, fluorescein; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline pH 7.2.

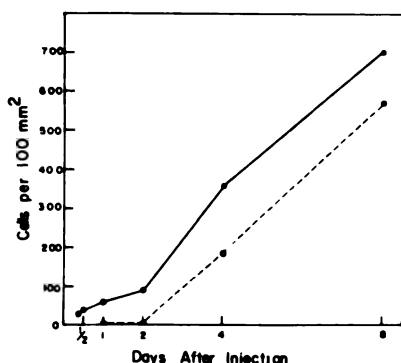


FIG. 1. Antibody-containing cells in the mouse spleen after a primary intravenous injection of HGG. Each point represents the mean cell count in the spleens of 3 mice. ●—●, total antibody-containing cell number at each time; ○----○, antibody-containing cell number in the white pulp.

Sections stained for mouse globulin (anti-MGG) revealed many more fluorescent cells in the red pulp of the mouse spleen at the early stage of the primary response than did the FITC-HGG conjugate. The MGG-containing cells were observed in the arteriolar areas, the subcapsular areas, and the areas near the edges of the follicles. Most of them, but not all, were found forming small clusters. Scarcely any fluorescent cells were found in the white pulp at this stage. At the later stages of the primary response we found many more fluorescent cells stained with anti-MGG-conjugate than with FITC-HGG conjugate. Anti-MGG conjugate revealed occasionally extra- or inter-cellular gamma globulin in paraffin sections.

The secondary response was about 10 times as extensive, and the changes twice as rapid, reaching their peak after 4 or 5 days. Here too the peak was reached in the white pulp, but the red pulp also contained more cells than during a primary response (Table I).

The exact location of the antibody-containing cells was not always easily assigned, because the observations were made under the fluorescent microscope with dark field illumination. However, the early cells were found in the splenic red pulp near the trabeculae. In the white pulp they appeared in the tissue sheathing the central artery and in the marginal zones around the follicles.

Although many sections were exposed to rhodamine-labeled antiBGz, no antigen remaining from injection was observed.

Table II presents cell counts for primary responses to both BGz and KLH. They also reflect the responses to various doses of BGz and KLH. It is of interest to note the coincidence that the optimal dose for BGz (molecular weight 540,000 daltons) was 50 μ g, and that for KLH (7,000,000 daltons) 10 mg, a roughly proportional number of molecules.

Discussion. The first change observed was the appearance of isolated large antibody containing cells 8 hr after the injection of HGG. These cells probably synthesize IgM as Hanaoka, Nomoto, and Waksman have demonstrated in the appendix of the rabbit (11).

The antibody-containing cells always appeared first in the red pulp before spreading into the white pulp. Positive cells appeared in the white pulp only if the dose of antigen was above a threshold which seemed to be different for various antigens. The injection of 1 μ g BGz produced no response in the white pulp at any time but 10 μ g elicited a measurable response by Day 8. For KLH, however, more than 100 μ g was required before any positive cells could be seen in the white pulp. The primary response began slowly, being barely detectable during the first two days, but then increasing from Days 2 to 4. And although there was a doubling of the small number of specific cells in the red pulp, those in the white pulp increased 20-fold during the same 2-day interval. Then, during the next 4 days, the increase slowed in the red pulp but continued in the white pulp. This type of distribu-

TABLE I. PRIMARY AND SECONDARY RESPONSES TO BGz.^a

BGz (days after injection)	Fluorescent cells/10 mm ²		Estimated mean/10 ⁶ cells	W/R ^b
	R	W		
1 ^c 2	4.6	0.3	48	0.07
5	60	8.2	682	0.14
12	33	68	1017	2.1
2 ^d 2	31	30	609	0.95
5	366	673	10060	1.83
8	102	80	1820	0.78

^a Three mice per group.

^b W/R is the ratio of the number of cells in the white pulp to those in the red pulp.

^c 0.1 mg heated BGz intraperitoneally.

^d 0.1 mg heated BGz in Freund's adjuvant, boosted 4 weeks later with 0.1 mg ip without adjuvant.

TABLE II. DOSE RESPONSES TO A FIRST INJECTION.

Dose (mg)	8 days BGz				12 days KLH			
	Fluorescent cells/10 mm ²				Fluorescent cells/10 mm ²			
	(R)	(W)	(W/R)	(Total/10 ⁶)	(R)	(W)	(W/R)	(Total/10 ⁶)
0.01	19.1	21.4	1.12	405				
0.05	22.1	142.1	6.46	1645				
0.1	14.7	70.7	4.82	854	4.4	0	0	41
0.5	28.8	69.4	2.42	982				
1.0	12.5	30.1	2.40	426	10.2	1.1	0.12	103
5.0	3.7	10.5	2.86	142				
10.0					14.4	31.8	2.2	463

tion of active cells occurred only if a relatively large dose of antigen was administered (5 mg HGG, 10 mg KLH). With smaller doses, the response in the white pulp appeared later or not at all.

Secondary responses to HGG and BGz antigens went through the same changes at a faster pace; the cells in the white pulp on the second day were 100-fold more numerous in absolute numbers and were equal to those in the red pulp. By the 5th day when the red pulp population had increased 10-fold, the white pulp cells had increased 20-fold. If this represents multiplication rather than recruitment, then the cells multiplied once every 9 hr.

During the primary response, cells positive for MGG appeared as a response to antigenic stimulation in more cells than those which contained specific antibody. This finding has been recorded by a number of observers, whose findings are summarized by Cazanave *et al.* (12). Some of these cells were found by them to contain globulin of the same idotype as the antibody, without *reactivity* for the antigen, a phenomenon not at present understood; perhaps it represents antibody of very low avidity.

At present it is not possible to understand the meaning of the changes described here, but they are clearly related to the differentiation of antibody-forming cells, and to the microenvironment associated with their development. The spread of antibody formation from the red to the white pulp during the primary antibody response may represent the migration of progeny of the cells from the red to the white pulp, or the recruitment of new cells by antigen as the response proceeds. Or, it might conceivably indicate the diffusion of a substance pro-

duced by the early cells which triggers the response of the later ones.

Summary. Enumeration of the cells in the mouse spleen making a specific response to antigenic stimulation indicates that the first detectable antibody appears in a few cells in the red pulp in the first 12 hr; later, such cells appear in the white pulp, and, though their number increases in both locations, the gain is more rapid in the white pulp, reaching a peak on the 12th day in the primary response, and on the 5th in the secondary, when there are two to four times as many cells there as in the red pulp. The response in the white pulp requires a higher threshold dose than that in the red pulp.

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Mechanism of Inhibition of Aminoglycoside and Polymyxin Class Antibiotics by Polyanionic Detergents (39478)

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Polyanionic detergents have been incorporated into blood culture media for many years. Sodium polyanetholsulfonate, known as Liquoid, has been the most extensively used and studied member of this class of compounds; it has been shown to possess antiphagocytic (2), anticomplementary (3), antiprotein (4), and anacardic activity (5, 6). SPS exhibits little antibacterial activity, with the family *Enterobacteriaceae* and genus *Staphylococcus* usually showing concentrations as high as 1.25% being inhibited, while the anaerobic streptococcus *Peptococcus anaerobius* (8) and occasional strains of *Neisseria meningitidis* (9) are inhibited by SPS. Sodium amylosulfonate, although recently introduced and not extensively studied, appears to have the same activity spectrum as SPS (10).

It appears to effectively obviate the action of cationic compounds. Positively charged antibiotics, such as aminoglycosides, polymyxins, and positively charged proteolytic enzymes as lysozyme and beta-lipoprolylase, are selectively affected (10). The mechanism of action by which polyanionic detergents inhibit antibiotics has not been determined directly. Studies in the literature have utilized an indirect indicator system, measurement of bacterial growth, to determine the effect of detergent on antibiotic activity. As a result, the mode of inhibition of antibiotics by polyanionic detergents remains undetermined. Accordingly, a study was undertaken to determine the mechanism of action; in lieu of an intermediate system a non-antigen-antibody diffusion system in agar gel, originally described by Kunin and Tupasi (11), was employed. Since the reaction milieu can have an effect on the activity of charged compounds, all experiments were conducted in free media.

Methods. Diffusion plates were made by

pouring 5 ml of 1.5% agarose in deionized distilled water into 60 × 15 mm plastic tissue culture dishes. Seven wells were cut using a template (Grafar Company, Detroit, Michigan); the six outer wells were 3 mm in diameter, the single inner well 4 mm in diameter. Inner wells contained a detergent concentration; the six outer wells contained a geometric dilution series of the tested antibiotics. All plates were used within 48 hr of pouring. Antibiotic and detergent solutions were added to the wells and the plates were incubated at both 4 and 22°C for 48 hr.

Antibiotics (Canalco, Rockville, Maryland), obtained as standard powders without preservatives, included the aminoglycosides streptomycin, kanamycin, gentamicin, and tobramycin, the polymyxins colistin and polymyxin B, and penicillin G. Antibiotic concentrations of 10,000, 5,000, 2,500, 1,250, 625, and 312 µg/ml were tested. Sodium polyanethol sulfonate (Hoffmann-LaRoche, Nutley, New Jersey), sodium lauryl sulfate (SLS) (Mann Research Labs., New York, New York), and disodium 4-dodecylated oxydibenzene sulfonate (Benax 2A1, Dow Chemical Company, Midland, Michigan) represented the polyanionic group. The nonionic and cationic detergents tested were tween 80 (Atlas Chemical Ind., Wilmington, Delaware) and benzalkonium chloride (Winthrop Labs., New York, New York) respectively. Detergent concentrations included 50,000, 12,500, 10,000, 6,250, 3,125, 1,560, and 780 µg/ml. All antibiotics and detergents were diluted in deionized distilled water and used within 2 hr of constitution.

All plates were observed at 1, 4, 18, 24, and 48 hr incubation. Lines of precipitation were recorded manually and photographically.

In order to establish that precipitation between antibiotic and polyanionic detergent

effectively reduced the amount of active antibiotic present in the milieu, a tube dilution system was constructed. Polyanionic detergents were geometrically diluted from 1 to 0.03% in distilled water. To each test tube containing a polyanionic detergent an equal volume of 0.5% antibiotic was added. After 4 hr of incubation at room temperature, the tubes were centrifuged. The supernatant was tested for free antibiotic and the pellet, after washing five times in distilled water, was resuspended and tested for bound antibiotic (12).

Results. All polyanionic detergents formed precipitin lines with streptomycin, kanamycin, gentamicin, tobramycin colistin, and polymyxin B (Table I). There were quantitative differences in both the lowest concentration of polyanionic detergents yielding a precipitin line and in the strengths of the precipitin lines. Neither SLS, SPS, nor Benax produced precipitin lines with penicillin. Figure 1 demonstrates a typical non-antigen-antibody precipitin line formed between a polyanionic detergent, SPS, and an aminoglycoside antibiotic, kanamycin. The precipitin line is analogous to that seen with Ouchterlony double diffusion between antigens and antibodies. All reactions between polyanionic detergents and aminoglycosides and polymyxins yielded only one precipitin band. In all cases precipitation resulted in the inactivation of antibiotic.

Most reactant concentrations produced visible lines of precipitation within 4 hr both at 22 and 4°C. Precipitin lines increased in density with time, reaching a maximum at 24 to 48 hr. All three polyanionic detergents were able to detect the lowest concentration antibiotic although the reaction was strongest with SLS. It thus appears possible that the detection of antibiotics may be pos-

sible by varying well diameters, the juxtaposition of the wells, and the buffer system. Benax was the least sensitive of the polyanionic detergents being able to detect 312 $\mu\text{g/ml}$ of aminoglycoside at a concentration of 10,000 $\mu\text{g/ml}$.

As would be expected, reactions were both stronger and more sensitive with the larger, higher molecular weight polypeptide, antibiotics colistin and polymyxin B. Here again SLS was the most active followed by SPS, while Benax was considerably less active (Table I).

Neither the nonionic detergent, tween 80, nor the cationic detergent, benzalkonium chloride, yielded visible precipitin lines with any of the antibiotics tested (Table I).

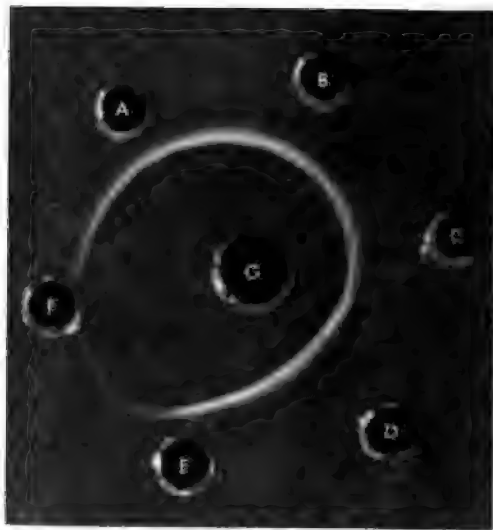


FIG. 1. Precipitation band formed between SPS and Kanamycin. Kanamycin concentrations are in the outer wells (A = 10,000 $\mu\text{g/ml}$, B = 5,000 $\mu\text{g/ml}$, C = 2,500 $\mu\text{g/ml}$, D = 1,250 $\mu\text{g/ml}$, E = 625 $\mu\text{g/ml}$, and F = 312 $\mu\text{g/ml}$) with 10,000 $\mu\text{g/ml}$ of SPS in the inner well (G).

TABLE I. INTENSITY OF THE PRECIPITATION REACTION BETWEEN DETERGENTS AND ANTIBIOTICS.^a

	STR ^b	KM	GM	TB	COL	POL	PEN
SPS	2+	2+	2+	2+	3+	3+	0
SLS	3+	3+	3+	3+	4+	4+	0
Benax 2A1	1+	1+	1+	1+	2+	2+	0
Tween 80	0	0	0	0	0	0	0
Benzalkonium chloride	0	0	0	0	0	0	0

^a 4+ represents the strongest reaction observed in this series (2500 $\mu\text{g/ml}$ POL and 2500 mcg/ml SLS).

^b Antibiotic abbreviations: STR—streptomycin; KM—kanamycin; GM—gentamicin; TB—tobramycin; COL—colistin; POL—polymyxin B; PEN—penicillin G.

ussion. It has been established that ionic surfactants are able to inhibit normal functioning of several biological systems and the activity of selected classes of antibiotics. Numerous studies using immunological markers have demonstrated the inhibitory effect of SPS on polymorphonuclear leukocytes (2, 13) and complement. Investigations concerning the reaction mechanism between polyanionic detergents and antibiotics have not been published. Following procedures originally presented by Nin and Tupasi (11) it was found that ionic detergents inhibit aminoglycoside and polymyxin classes of antibiotics by reacting directly with them to form a precipitate. All three polyanionic detergents, although demonstrating quantitative differences in their inactivating abilities, form precipitation lines with all members of antibiotic classes.

Other than the nonionic detergent, tween 80, the cationic detergent, benzalkonium chloride, was found to react with any of the antibiotics tested. It would appear that precipitation, and concomitantly inactivation, is a direct result of the interaction of a positively charged detergent with a positively charged antibiotic, to form an insoluble polymer. Apparently at least two positively charged amino groups on the antimolecule are available for bonding with the negatively charged groups on the detergent to create a lattice-like structure which then forms a precipitate. Unlike other detergents utilized to inactivate aminoglycoside antibiotics, such as the addition of calcium to bacteriological media, which indirectly affects the antibiotic by changing bacterial cell wall permeability (15), polyanionic detergents inactivate aminoglycosides and polymyxins directly, with no effect on other antibiotics.

It appears theoretically possible (16) to develop a quantitative antibiotic assay based on the formation of non-antigen-antibody precipitation lines since only one band of precipitation is formed and the density and position of the band vary in direct relationship with the concentration of the reactants. Summary. Polyanionic detergents, the

most widely used of which is sodium polyanethanol sulfonate (SPS), inhibit polymorphonuclear leukocyte, complement, lysozyme, and antibiotic activity. SPS has been utilized for years in the culture of blood for bacterial pathogens. Utilizing an agarose gel double diffusion system it was ascertained that polyanionic detergents, as represented by SPS, sodium lauryl sulfate, and disodium 4-dodecylated oxydibenzene-sulfonate, inactivate antibiotics by combining directly with them to form a precipitate. Only the positively charged aminoglycoside and polymyxin classes of antibiotics were affected. Neither nonionic nor cationic detergents interacted with aminoglycoside antibiotics. It would appear that a polymer is formed with both the polyanionic detergent and antibiotic each at least divalent. The reaction is independent of and does not interfere with bacterial growth.

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Zinc-Binding Protein: Relationship to Short Term Changes in Zinc Metabolism^{1,2} (39479)

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A variety of functions has been suggested for metallothionein (MTN), a low molecular weight cytoplasmic metalloprotein. These include roles in detoxification of Cd, Hg or Zn (1, 2), hepatic storage of Zn and Cu (3), Cu absorption (4), and Zn metabolism (5, 6). Recently we proposed a dual function for a Zn-binding protein (ZnBP), which is similar to MTN, in the homeostatic regulation of Zn metabolism. Synthesis of both mRNA and ZnBP appeared to be required for plasma Zn uptake into liver cells where ZnBP appeared to serve as a storage protein (7). Furthermore, we found that intestinal ZnBP bound newly absorbed dietary Zn and suggested the protein may serve to antagonize absorption by competing for Zn with an intracellular chelator in mucosal cells. In order for ZnBP to serve these functions, its synthesis and degradation must be readily responsive to a metabolic signal. Previously we demonstrated that parenterally administered Zn resulted in changes in serum Zn concentrations prior to changes in liver or intestinal ZnBP synthesis (8). It therefore appeared plausible that serum Zn may serve as a short-term metabolic modulator of Zn metabolism via its influence on ZnBP synthesis. The experiments described here demonstrate that the short-term response of liver and intestinal ZnBP formation is related to fluctuations in serum Zn which can be brought about by changes in dietary Zn level as well as parenterally administered Zn.

Materials and methods. Weanling male rats (Sprague-Dawley, Madison, Wisconsin) were housed individually in stainless

steel cages and fed *ad lib.* a standard laboratory chow that contained 50 ppm Zn until a weight of 150 g was attained, at which time the rats were used for experiments. For the studies involving the parenteral Zn dose, 25 μ mole of Zn (as ZnSO₄ in 0.9% saline) was injected (ip) and 0, 8, 24, or 48 hr later the rats were killed by decapitation and the blood was collected. The livers and intestinal mucosa from each rat within each treatment group were pooled and homogenized in 0.25 M sucrose-10 mM Tris-HCl (pH 8.6). The 105,000 g supernatant was applied to a column (2.6 \times 50 cm) packed with Sephadex G-75 and eluted as described previously to isolate ZnBP (7). Chromatographic fractions and serum were measured for Zn content by atomic absorption spectrophotometry.

In studies involving dietary manipulations, the rats were housed as above except they were given resin demineralized H₂O and fed either a Zn deficient diet or the same diet supplemented (150 ppm Zn) with ZnSO₄. The Zn deficient diet (<1 ppm Zn) was that described previously, except that spray-dried egg white was used as the protein source and Zn was omitted from the mineral mix (9). The rats were depleted of Zn by feeding the Zn deficient diet for a 24 hr period. Subsequently they were fed the 150 ppm Zn-supplemented diet for 24 hr and were then fed the Zn deficient diet for an additional 24 hr period. One-third of the rats were killed following each of the three feeding periods. The serum, liver, and intestinal mucosa were collected, prepared and analyzed as described above.

In a third study, rats were fed the Zn deficient diet for 7 days after which time the animals were fed the Zn deficient diet supplemented with 0, 25, 75, or 150 ppm Zn (as ZnSO₄) for 24 hr. The serum, liver, and intestinal mucosa were collected, prepared, and analyzed as described above.

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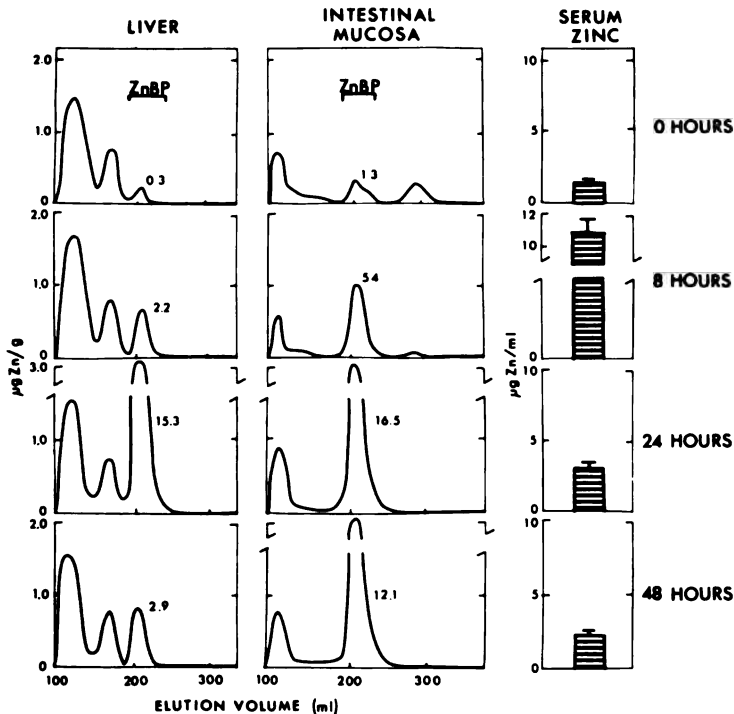
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and discussion. The Sephadex G-75 profiles of both liver and intestinal mucosal cytosol and the corresponding Zn concentration from rats fed a stock diet (50 ppm Zn) are shown. Under these conditions serum Zn is nearly constant (1.2 $\mu\text{g}/\text{ml}$). The liver fractionated into 3 Zn-containing fractions (left, top profile). Peak I represented low mol wt Zn-containing proteins. Peak II was shown to possess both superoxide dismutase and carbonic anhydrase activity and the third peak, which corresponded to a molecular weight of 6,000–12,000 daltons, designated as ZnBP. Intestinal cytosol (right, top profile) also fractionated into 3 Zn-containing peaks. In this fraction, eluted near the void volume of the column, again comprised the high mol wt Zn-containing proteins, however the second peak in the mucosal profile corresponded to ZnBP. Both liver and intestinal ZnBP were found to have identical

chromatographic properties on a standardized G-75 column. The third Zn peak in the mucosal cytosol profile corresponded to a very low mol wt (<1,000 daltons) Zn-chelating complex that has been implicated to function in intestinal Zn absorption (7, 11).

The remaining profiles in Fig. 1 show changes in Zn distribution among the chromatographic fractions from liver and mucosal cytosol and the serum Zn concentration at 8, 24, or 48 hr following parenteral Zn administration. At 8 hr postinjection serum Zn was significantly elevated above control levels ($P < 0.01$) to 11.0 $\mu\text{g}/\text{ml}$ and both liver and intestinal ZnBP showed a modest accumulation of Zn. However, by 24 hr serum Zn had declined significantly ($P < 0.01$), while Zn bound to cytoplasmic ZnBP had increased in both tissues. These data support our suggestion that ZnBP synthesis may be required for the uptake of Zn from the blood into these tissues (7, 8). By 48 hr following the Zn injection ZnBP-



Sephadex G-75 elution profiles of cytosol-bound zinc from liver and intestinal mucosa and the serum concentration of zinc in adequate rats. The rats were killed 0, 8, 24, and 48 h after an injection (ip) of 25 μmole ZnSO_4 . ZnBP is the zinc-binding protein fraction and the number next to each ZnBP peak is the total zinc ($\mu\text{g Zn/g}$ fresh tissue). Fractions eluted between 100–140 ml comprise Peak I of cytosol from liver and fractions eluted between 150–180 ml comprise Peak II of liver cytosol. Each serum Zn value is the mean \pm SEM of three rats.

bound Zn had declined in the liver, but not in mucosa, from the value obtained at 24 hr and the serum Zn content decreased to within normal levels.

We investigated the nature of the ZnBP response in animals fed diets of varied Zn content for short periods of time (24 hr). The changes in chromatographic behavior of liver and mucosal cytoplasmic Zn from rats subjected to a Zn depletion-repletion-depletion regime achieved by feeding either the Zn deficient diet (<1 ppm Zn) or the same diet supplemented with 150 ppm Zn is shown in Fig. 2. Initially (Day 0) a small quantity of Zn was bound to intestinal and liver ZnBP and serum Zn levels were within normal limits. This agreed with previous data that demonstrated when steady state levels of dietary Zn are maintained large amounts of ZnBP are not found (5-8, 10, 12). After the Zn deficient diet was fed for 24 hr (Day 1) the major change in soluble

Zn was that bound to ZnBP. Both liver and mucosal ZnBP-bound Zn declined to trace amounts and serum Zn decreased to 0.4 $\mu\text{g}/\text{ml}$. If these Zn depleted animals were subsequently fed the Zn supplemented diet (150 ppm Zn) for 24 hr (Day 2), there was a substantial increase in the Zn content of both liver and intestinal mucosal cytosol. This increase occurred almost entirely in ZnBP-bound Zn and was approximately equal in both tissues. The serum Zn concentration was significantly ($P < 0.01$) elevated, to 5.2 $\mu\text{g}/\text{ml}$, which was five times that found in control rats. If the rats were subsequently fed the Zn deficient diet for an additional 24 hr (Day 3), the major decline in soluble Zn was accounted for by ZnBP-bound Zn. Serum Zn levels declined to near normal limits.

Comparison of the data in Figs. 1 and 2 indicated that the magnitude of the response of Zn accumulation by ZnBP was reasona-

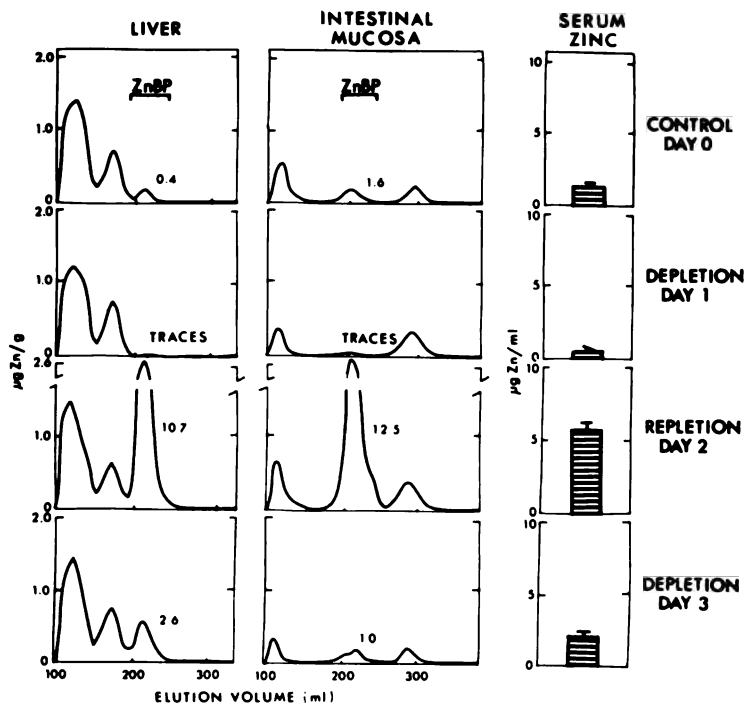


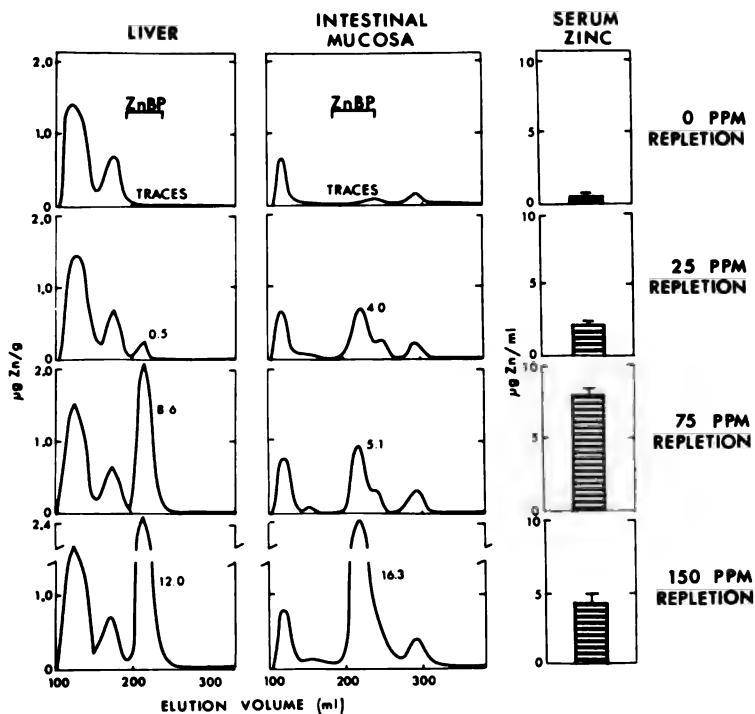
FIG. 2. Sephadex G-75 elution profiles of cytosol-bound zinc from liver and intestinal mucosa and the serum zinc concentration of rats. The rats were fed a Zn deficient diet for 24 hr (Day 1, Depletion), then were fed a Zn sufficient diet for 24 hr (Day 2, Repletion), and finally were fed the Zn deficient diet for 24 hr (Day 3, Depletion). ZnBP is the zinc-binding protein fraction and the number next to each ZnBP peak is the total ZnBP-bound Zn (μg Zn/g fresh tissue). Fractions eluted between 100-140 ml comprise Peak I of cytosol from both tissues and fractions eluted between 150-180 ml comprise Peak II of liver cytosol. Each serum Zn value represents the mean \pm SEM of three rats.

comparable whether Zn was administered parenterally or was fed. Zn absorption was found to increase after feeding a Zn deficient diet (12). The large accumulation of both liver and intestinal ZnBP following depletion and repletion as well as the elevated serum Zn levels are indicative of normal Zn absorption from the diet. The relative Zn content of the molecular weight proteins were not affected by the changes in dietary Zn. Therefore ZnBP is the cytoplasmic protein component that is dynamically correlated to changes in serum Zn concentrations.

A series of experiments were conducted to investigate the effects of a 7 day depletion followed by a 24 hr repletion where varying levels of supplement were fed. Accumulation of Zn by liver and intestinal ZnBP appeared to follow similar courses for the varying Zn levels (Fig. 3). The increase in liver ZnBP-bound

Zn was found to be exponential. In the intestine there was a moderate increase in ZnBP-bound Zn up to 75 ppm of Zn supplementation. When 150 ppm was fed, there was a substantial accumulation of Zn in mucosal ZnBP and a concomitantly smaller increase in serum Zn compared to when 75 ppm was fed. This latter observation would correspond to increased hepatic uptake and decreased absorption (7).

Previous experiments have shown that changes in serum Zn concentration, in response to parenterally administered Zn, preceded changes in tissue ZnBP-bound Zn levels. This indicated that perhaps serum Zn served as a modulator of Zn metabolism by initiating the appearance of ZnBP (7). High and near-toxic levels of dietary Zn have been shown to produce elevated levels of liver MTN (6). Thus MTN was previously believed to serve a detoxification role. The present data demonstrate that changes in



3. Sephadex G-75 elution profiles of cytosol-bound zinc from liver and intestinal mucosa and the serum concentration of rats. The rats were fed a Zn deficient diet for 7 days and then were fed the same diet but supplemented with 0, 25, 75, or 150 ppm Zn as ZnSO_4 for 24 hr. ZnBP is the zinc binding protein fraction and the area under each ZnBP peak is the total ZnBP-bound zinc ($\mu\text{g Zn/g}$ fresh tissue). Fractions eluted between 150–180 ml comprise Peak I of cytosol from both tissues and fractions eluted between 150–180 ml comprise Peak II of cytosol. Each serum Zn value represents the mean \pm SEM of three rats.

serum Zn concentrations and liver and intestinal ZnBP-bound Zn are rapid and significant in response to subtle changes in dietary Zn level. These results suggest that ZnBP is part of the mechanism that regulates the daily metabolism of zinc in animals. Preliminary evidence from our laboratory as well as others (10) suggests that ZnBP has characteristics identical to those of MTN. The remarkable similarity in cysteine content, metal to protein ratio, dimorphism when subjected to DEAE-ion exchange chromatography and a lack of absorbance at 280 nm are all consistent with the known characteristics of MTN. An amino acid analysis of a zinc binding protein obtained from rats given a parenteral zinc load has shown conclusively that metallothionein is the protein which was isolated (10). It therefore appears that MTN is the protein which responds to the treatments described above.

Summary. The short-term changes in hepatic and intestinal mucosal zinc binding protein (ZnBP) bound Zn and in serum Zn concentration were investigated following either parenterally administered Zn or changes in dietary Zn. Both treatments resulted in similar changes in ZnBP-bound Zn. Repletion of Zn depleted rats resulted in increased hepatic and intestinal ZnBP-bound Zn and subsequent depletion decreased ZnBP-bound Zn in both tissues. These results demonstrate that ZnBP formation and degradation responds readily to fluctuations in dietary Zn level. Serum Zn

was correlated to the appearance of in both tissues and could be the mesial signal that influenced the amount of associated with ZnBP. The data suggest ZnBP serves a regulatory function in metabolism. Finally preliminary evidence suggests that ZnBP may be identical to metallothionein.

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Rabbit Platelet Aggregation by Tuberculin¹ (39480)

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A wide variety of materials has been used to induce platelet changes resulting in aggregation and loss of internal contents (1-3). Many of the substances used are capable of bringing about changes by mechanisms involving direct interaction with a receptor site on the surface. Other materials require the presence of complement, and in some cases, additional plasma cofactors for initiation of aggregation and release reactions. The present communication reports on platelet-aggregating activity by tuberculin, a substance which appears to irreversibly aggregate platelets via a complement-dependent mechanism.

Materials and methods. Platelets. Blood was obtained by cardiac puncture from adult male and white rabbits purchased locally. Blood was drawn into plastic syringes containing one-tenth volume of 3.8% trisodium citrate or enough ethylene-diamine tetraacetic acid (EDTA) or ethylene-glycol tetraacetic acid (EGTA) to produce a final concentration of 10 mM (calculated from hematocrit determinations). Platelet-rich plasma (PRP) was obtained after centrifugation of the blood at 150g for 20 min at room temperature. Platelets in PRP were counted in duplicate by the method of Lasker (8). For some experiments, albumin-coated platelets were prepared according to the procedure of Walsh (9).

Tuberculin. Lot numbers 97477B and 97477L were obtained from Parke-Davis Company, Detroit, Michigan. Confirmations of the lyophilized powdered form of purified protein derivative (PPD) w/w, nucleic acid content 9.6% w/w, carbohydrate 42.2% w/w. Stock solution 1 mg/ml were prepared in saline.

Aggregometer. Platelet aggregation was measured turbidometrically on a Chrono-

log aggregometer (model No. 300, Chronolog Corp., Broomall, Pennsylvania) attached to a Heath Servo Chart Recorder (Model SR-201A). The baseline of the recordings was established with PRP adjusted to a concentration of 500,000 platelets/ μ l and the scale adjusted to give maximum deflection at the optical density of platelet-poor plasma (PPP). For studies of aggregation, PRP was placed in a siliconized cuvette and inserted into a temperature control holder with controlled magnetic stirring at 1200 rpm. A baseline was recorded for 5 min and the light transmission through the cell suspension recorded.

Complement inactivation and depletion. Heat inactivation of complement was accomplished by heating PPP to 50°C for 30 min. Heated plasma was centrifuged to remove precipitated fibrinogen prior to resuspension of fresh platelets. Hydrazine hydrate (Fisher Scientific Co., Medford, Massachusetts) adjusted to pH 7.5 was incubated with plasma at a final concentration of 5 mM for 60 min, the mixture dialyzed overnight at 4°C against 0.15 M NaCl with 5.5 mM sodium citrate (10) and added to fresh platelets. Plasma samples were incubated with four units of Cobra Venom Factor (CoF) at 37°C for 30 min. The purified material, the product of Cordis Laboratories (Miami, Florida), was suspended and diluted in distilled water immediately prior to use. Zymosan (Z) obtained from Sigma Chemical Company, St. Louis, Missouri, was preincubated with PPP at a concentration of 2 mg/ml (11) for 2 hr at 37°C.

Results. Recorder tracings of platelet aggregation by tuberculin. Typical aggregometry tracings of tuberculin-induced aggregation in stirred PRP are shown in Fig. 1. Aggregation responses were dose dependent for all concentrations. The first phase of the recorded reaction was a brief 1-2 min lag which diminished with increasing concentrations of tuberculin. During this period

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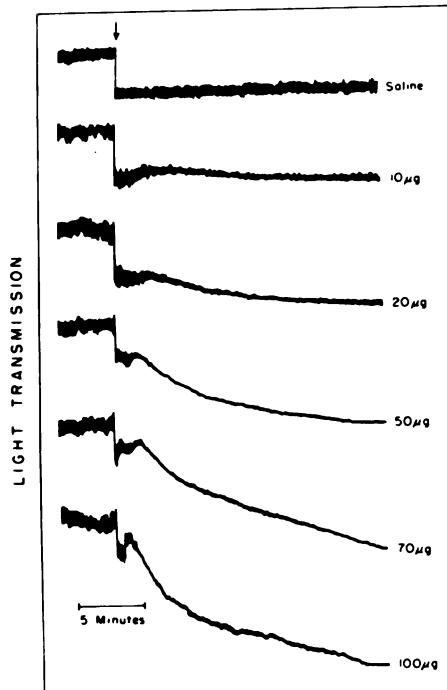


FIG. 1. Aggregometer tracings of tuberculin-induced aggregation in stirred citrated rabbit platelet-rich plasma. Tuberculin was added to a final concentration of 10, 20, 50, 70, and 100 μg per ml and a portion of the same plasma was inoculated with a similar volume of saline.

there was no change in either the slope of the curve or the vertical oscillations produced by the stirred discoid platelets. Microscopic examination of samples, however, revealed that small clumps consisting of 12–15 platelets had formed at this time. For tuberculin concentrations greater than 10 $\mu\text{g}/\text{ml}$, this phase was followed by an irreversible decrease in absorbance and reduction in amplitude of oscillations. Large aggregates with marked alterations in morphology were seen under phase contrast microscopy (Fig. 2). Many of the platelets contained in the clumps were transformed to irregularly swollen forms having greatly reduced refractility. Some of the platelets appeared to have fused and could not be identified as distinct cells. The morphological alterations were similar to those platelet changes collectively described as viscous metamorphosis (1). There was essentially no difference in sensitivity to tuberculin of

platelets from different rabbits and aggregation was not observed in PRP incubated with saline. Platelet aggregation was observed following addition of tuberculin to human citrated PRP prepared from blood.

Complement participation in the response. The initial lag period between addition of tuberculin and appearance of platelet aggregation suggested an action requiring plasma factors. Tuberculin failed to initiate aggregation when incubated with washed platelets suspended in Tyrode's solution. Addition of small amounts of PPP to the washed platelets restored the aggregating activity.

The extent of complement participation was assessed by measuring aggregation of platelets in plasma which had been treated to inactivate or deplete complement. CoF, which inactivates C3–C9 by blocking reactions leading to the assembly of the alternate pathway C3 convertase (12) inhibited the ability of the plasma to support aggregation response. Chemical inactivation of C3 by pretreatment of plasma with zinc (13) similarly inhibited the response. Platelets suspended in plasma heated at 50°C for 30 min displayed minimal aggregation upon addition of tuberculin. A degree of activity was restored by mixing equal volumes of heated and hypotonic treated plasma. Removing proteins which activate the alternate complement pathway (14) by preincubation of plasma with Z (15) abolished the ability of tuberculin to initiate platelet aggregation.

Differential chelation of divalent cations using EGTA was employed to help determine which complement pathway participated in the platelet-tuberculin interaction. EGTA-PRP provides appreciable concentrations of ionized magnesium but very low ionized calcium and is therefore incapable of sustaining activation of the alternative pathway (16). With EDTA as an agent, the concentration of both calcium and magnesium ions is too low to support activation of either complement pathway. Aggregometer tracings produced in EDTA-PRP and EDTA-PRP are shown in

agulation with EGTA permitted a delayed aggregation response characterized by a lag ranging from 15 to 35 min. Aggregation was not observed in plasma anticoagulated with EDTA.

Discussion. Repeated experiments have demonstrated that tuberculin initiates aggregation of rabbit platelets. The dependence of the reaction upon plasma factors is evident by the fact that aggregation does not occur when tuberculin is added to albumin-coated platelets suspended in salt solution. Requirement for C3 is indicated by the absence of a response following addition of zine. This treatment may abrogate the response by preventing complement-dependent adherence reactions which are associated with aggregation of nonprimate platelets (17). In addition, either hydrazine or Z may prevent aggregation by aborting development of the lytic reaction mediated by terminal complement components.

Activation of C3 may occur via the classical complement pathway initiated by antibody-antigen complexes or by the ACP. The ACP can be activated *in vitro* by Z (15) and by a disaccharide (18). The present findings indicate that tuberculin can utilize the ACP pathway for the consumption of complement previously heated to 50°C for 30 min to support a rapid aggregation response; whereas a mixture of heated and zine-treated plasma does not. Heat and zine-sensitive factors required for activation of the ACP by Z have been identified as a sialic acid-rich β -glycoprotein (Factor B) and C3 (Factor A), respectively (19). The action of Z with human platelets has previously been shown to be a multisite reaction which also requires the presence of fibrinogen (14).

Rabbit platelets do not respond to tuberculin in plasma-PRP whereas a delayed and diminished aggregation reaction was observed in plasma chelated with EGTA. It has previously been shown that in EGTA-PRP, immunocomplexes and Z induce a similar pattern of aggregation which was explained in terms of the action of the chelator (11). The concentration of magnesium in EGTA plasma is suboptimal for full activation of

the ACP and the lack of ionized calcium would prevent aggregation reactions associated with the release of platelet adenosine-5'-diphosphate.

The inhibition of the aggregation reaction by adsorption with Z does not exclude the possibility that the response may also involve tuberculin activation of the classical complement pathway. Z has been reported to activate and consume C1-C4 (20) at a magnitude which may be sufficient to prevent tuberculin utilization of the classical pathway. Activation of C1 could occur by direct proteolytic cleavage or through interaction of tuberculin with complement fixing antibody. We have been unable to detect precipitating antibody by immunodiffusion, and tuberculin skin tests performed at the conclusion of the experiments were uniformly negative for immediate or delayed reactions. However, conventional serological procedures may not reveal the presence of low avidity antibody (21) and positive skin reactions do not correlate with the presence of antibodies to mycobacterial antigens (22). Although the rabbit is not a natural host for the tubercle bacillus, rabbit plasma may contain tuberculin-reactive immunoglobulin through immunization with cross-reactive antigens. In this regard, the sera of unimmunized and uninfected animals have been shown to induce immune adherence of *Mycobacterium tuberculosis*; the activity is interpreted as being due to low levels of natural antibody acting in conjunction with complement (17).

The present studies permit speculation that complement activation by antigens of the tubercle bacillus may be of some pathobiologic significance with respect to the development of tuberculosis lesions *in vivo*. There is a sizeable block of information that establishes a major role for fluid phase by-products of complement component interaction in the development of inflammatory lesions (23). The pathological changes which accompany tuberculosis in species bearing platelets sensitive to complement activation may be partially due to aggregation and release reactions. Rabbits, for example, consistently demonstrate thrombosis of small blood vessels during experimental

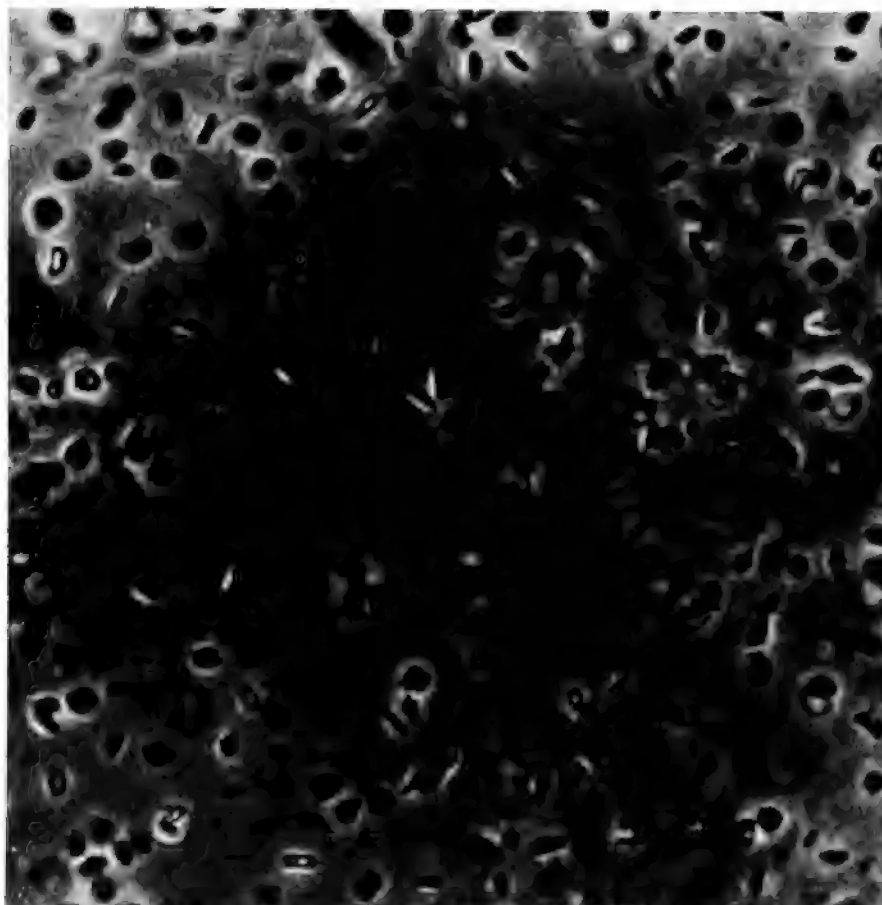


FIG. 2. Phase-contrast photomicrographs. A. Platelets in citrated plasma with saline, B. Platelets in citrated plasma incubated with 100 $\mu\text{g/ml}$ tuberculin for 5 min at 37°C. Phase-contrast, $\times 1925$.

TABLE I. THE EFFECTS OF VARIOUS PLASMA TREATMENTS ON THE ABILITY OF TUBERCULIN^a TO INDUCE PLATELET AGGREGATION.

Plasma treatment	Platelet aggregation ^b (0 to +++)
Untreated control	++++
Cobra venom factor (4 units)	0
Hydrazine (5 mM)	0
Heated (50 C/30 min)	+
Heated and Hydrazine (combined) ^c	+++
Zymosan ^d	0

^a Treated plasma preparations plus platelets were incubated for 30 min with tuberculin at a final concentration of 100 $\mu\text{g/ml}$.

^b Platelet aggregation was determined turbidometrically and by phase contrast microscopy.

^c Plasma containing equal volumes of heated and hydrazine treated plasma.

^d Zymosan was removed by sedimentation at 650g and platelets added.

tuberculosis (24). The induction by tuberculin of small platelet plugs could contribute to the development of thrombi and associated necrosis.

Summary. Tuberculin irreversibly aggregates rabbit platelets suspended in their native plasma. Phase contrast microscopy shows shape change and tightly packed aggregates. Inactivation of complement components by heat, hydrazine, cobra venom factor, and zymosan inhibit the reaction. Recombination of heat and hydrazine-treated plasma restored the capacity of tuberculin to initiate platelet aggregation. A delayed and diminished pattern of aggregation was observed in plasma chelated with EGTA while no response was observed in plasma anticoagulated with EDTA. The findings indicate that tuberculin can cause

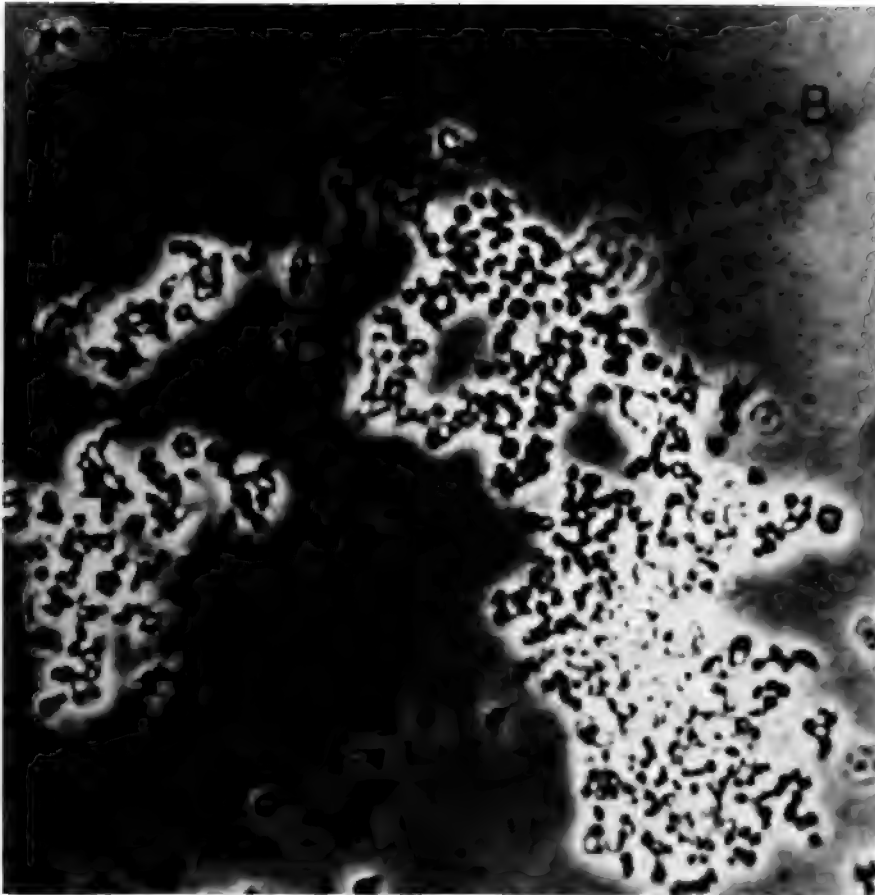
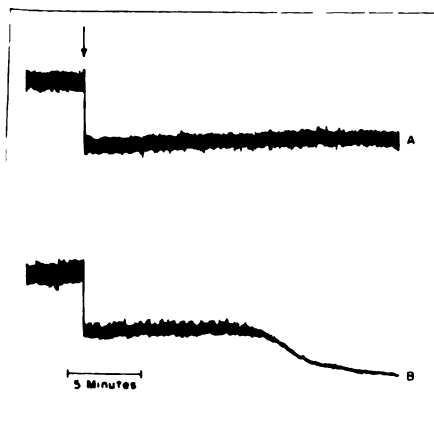
FIG. 2. *Continued.*

FIG. 3. Effect of chelation with EDTA and EGTA on tuberculin induction of platelet aggregation. Curve A: EDTA-PRP containing 100 $\mu\text{g/ml}$ tuberculin. Curve B: EGTA-PRP containing 100 $\mu\text{g/ml}$ tuberculin.

aggregation of rabbit platelets through a complement dependent mechanism, possibly utilizing the alternate pathway.

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Estrogen-Mediated Production of Type C Viral DNA Polymerase in Aged NIH Swiss Mouse Uteri (39481)

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al years our laboratory has been e phenomenon of estrogen-mencement of murine leukemia virus-synthesis in the uteri of ovariectomized (1-5). Viral proteins examined (NA-directed DNA polymerase "transcriptase") and p30, the major protein which bears the group-specific determinants. We have expression of viral markers in uterus is strain-dependent (3) level of viral proteins is dependent on the relative biological potency of estrogen (5). We now report a study of the ability of young ovariectomized NIH Swiss mice to respond to estrogen by production of viral markers in the uterus.

and methods. Animals. All mice were Swiss females bilaterally ovariectomized at age 14-22 days. Animals in the young group were 2-5 months old and in the aged group 2.0-2.5 years old. Estrogen-treated mice were injected intramuscularly with 1.0 μ g of 1,3,5(10)-estratrien-3-one in peanut oil at 0 and 24 hr and at 48 hr by cervical dislocation. **Preparation and assays.** Uterine homogenates were prepared in 0.5 M KCl containing Triton X-100 as described (4). Supernatants were analyzed by ultracentrifugation velocity gradients in 10% sucrose for RNA tumor virus-type C DNA polymerase using polyoma (dT) as template-primer (4). Murine leukemia virus p30 antigen was assayed by radioimmune precipitation as double antibody technique (6). Antisera were prepared in goat antiserum against Rauscher murine leukemia virus supplied by Dr. Roger Hunter at Huntington Laboratories. The

second antibody was rabbit anti-goat IgG from Meloy Laboratories. Highly purified p30 was obtained from Dr. Wade Parks, Meloy Laboratories. DNA was determined by the method of Hatcher and Goldstein (7), and protein was measured according to Bramhall *et al.* (8).

Histology. Uteri and adrenal glands were dissected from old and young animals, fixed in 10% formalin and 70% ethanol, and paraffin sections were stained with hematoxylin and eosin, Masson's trichrome, or Sudan black.

Results. Aged NIH Swiss mice, even though bilaterally ovariectomized before sexual maturity and untreated with estrogen, had much larger uteri than younger animals (46 vs 6 mg, respectively, Table I). In fact, unstimulated aged mice had uterine weights approximately equal to those of the estrogen-stimulated young mice. The larger uteri in aged animals may have resulted from estrogen produced by the adrenals or elsewhere. Young mice responded to estrogen treatment with a six- to sevenfold increase in uterine weight while older animals had slightly less than a twofold increase (Table I). The amount of weight gained, however, was about the same for both groups (35 and 43 mg, respectively).

There was a fivefold increase in total protein per uterus in young mice in response to stimulation while in aged animals the increase was only twofold (Table I). The actual amount of increased protein, however, was nearly the same in both groups (1.7 and 2.2 mg). DNA per uterus increased threefold upon stimulation in young animals but did not increase in aged mice.

Estrogen treatment of young, ovariectomized mice increased uterine p30 protein from 0.020 ng p30 per μ g protein in unstimulated to 0.051 ng p30 per μ g protein in stimulated mice (Table I). On the basis of

TABLE 1. EFFECT OF ESTROGEN TREATMENT ON VARIOUS UTERINE PARAMETERS IN YOUNG AND AGED NIH SWISS OVARECTOMIZED MICE.^a

	Unstimulated	Stimulated
p30 (ng p30/ μ g protein)		
young (3)	0.020	0.051 ^b
old (4)	0.033	0.044 ^b
p30 (total ng per uterus)		
young (3)	6.6	103.0 ^b
old (4)	89.0	215.0 ^b
Uterine wt. (mg)		
young (3)	5.9	41.3 ^b
old (4)	46.5	89.4 ^b
Total uterine protein (mg)		
young (3)	0.4	2.1 ^b
old (4)	2.4	4.6 ^b
Total uterine DNA (μ g)		
young (4)	367	808 ^b
old (4)	308	350

^a Mice in the stimulated group were injected intramuscularly with 1.0 μ g of estradiol-17 β at 0 and 24 hr and killed at 48 hr. Determinations were made on uterine homogenates as described in the text. The data were analyzed as paired samples (unstimulated and stimulated pools assayed simultaneously). The number of sample pairs is in parentheses.

^b Significantly greater than value for unstimulated at 95% confidence level.

total p30 per uterus the difference between unstimulated and stimulated was greater (7 and 103 ng, respectively). The aged animals also showed increased p30 specific activity in response to estrogen treatment (0.033 ng/ μ g protein in unstimulated vs 0.044 ng/ μ g protein in stimulated) and an approximately 2.5-fold increase (89 ng to 215 ng) in total p30. As with uterine weight and total protein values, the total amount of p30 increase in response to estrogen was about the same for both groups (96 ng for young and 126 ng for old).

Figure 1 compares glycerol sedimentation velocity gradient profiles of viral-type polymerase from supernatants of uteri from untreated and estrogen-treated, ovariectomized NIH Swiss mice of the two age groups. In the young animals marked increase in DNA polymerase upon estrogen administration is evident, while the aged group shows no change. A mixing experiment was conducted in which equal volumes of supernatants from aged and young mouse uteri were mixed and run on glycerol gradients. The resulting profile of DNA polymerase activity was essentially superimposable on the profile obtained by plotting the

sum of the corresponding fractions of gradients run separately with each supernatant (data not shown). This experiment suggests that the low enzyme activity in the old mice is not due to a soluble inhibitor present in excess in the supernatant.

In a previous study (5), we determined that concentrations of viral marker proteins in the uteri of ovariectomized NIH Swiss mice were greatest 48 hr after a single intramuscular injection of estrogen. The time sequence may be different in the aged mice. To test this possibility, a group of animals was given estrogen on Days 0, 4, 6, 8, 11, and 12 and sacrificed on Day 13. Glycerol gradient profiles of polymerase from uteri of these animals were not different from those of mice given estrogen only at 0 and 24 hr and killed at 48 hr.

When preparing homogenates we noticed

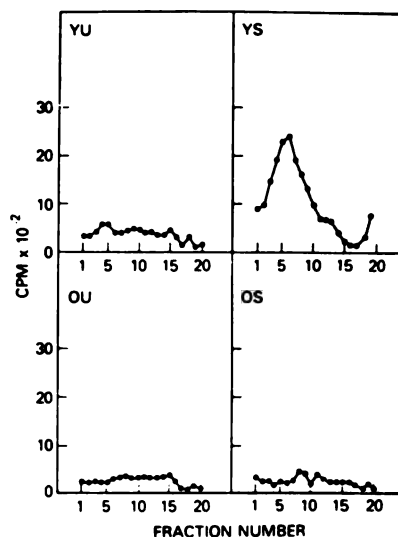


FIG. 1. Glycerol sedimentation velocity gradient profiles of viral-type DNA polymerase from old and young mouse uterine tissue. Portions of high speed supernatants (100 μ l) from 25% homogenates of uteri from young unstimulated (YU), young estrogen-stimulated (YS), old unstimulated (OU), and old estrogen-stimulated (OS) NIH Swiss ovariectomized mice were layered onto 4 ml 10 \rightarrow 30% glycerol gradients containing 500 mM KCl, 2 mM dithiothreitol, 10 mM Tris-HCl (pH 8), 2 mM magnesium acetate, and 0.05% Triton X-100. Gradients were centrifuged 15 hr in an SW56Ti rotor, the bottom of each tube was punctured and drops collected. Fractions were assayed for DNA polymerase activity using poly(A) \cdot oligo(dT) as template-primer as previously described (4).

the uteri from aged mice were larger than uteri from young, estrogenated mice. Histological examination of uteri of young, intact (nonovariectomized) mice (Fig. 2B) showed the absence of hyperplasia of all the tissue layers. Uteri of the aged mice (Fig. 2C), whether estrogen-treated or not, did not show the atrophic changes seen in the estrogen-withdrawn organ (Fig. 2A). All tissue

in the uteri of aged animals were as plastic as in the intact organ. However, in contrast to the uterus of an intact animal, numerous cysts were present in the endometrial stroma, and there was an increase in the amount of connective tissue in both the endometrium and the myometrium (Masson's trichrome stain, not shown). Using fluorescent antibody techniques and electron microscopy we have previously shown localization of viral proteins in the glandular and luminal epithelial cells (9).

In many of the aged mice we observed enlarged adrenals, and some appeared malignant, with destructive invasion of the kidney. Histological comparison of adrenals from aged and young, ovariectomized mice indicated the presence of adrenocortical carcinoma in the aged animals. Characterization of these tumors will be reported elsewhere.

Discussion. We have previously shown that uteri from ovariectomized NIH mice respond to estrogen by increased synthesis of a DNA polymerase which used λ -oligo(dT) as template-primer. The enzyme will also copy the poly C strand of λ -oligo(dG). Although of higher molecular weight than the polymerase from murine leukemia virus, the uterine enzyme cross-reacts in enzyme inhibition assay with specific antisera made against purified Rauscher virus polymerase. We suggested that the enzyme of mouse might be a precursor of the DNA polymerase associated with mature viral particles.

To determine the function of the viral-type RNA-dependent DNA polymerase we have determined that the function in the mouse uterus is unknown, the biological significance, if any, of the failure of aged mice to produce the enzyme in response to estrogen is not clear. However,

the lack of increased levels of DNA in the uteri of older animals after estrogen treatment may implicate this enzyme in DNA replication. The presence of elevated p30 levels in the absence of increased viral-type DNA polymerase suggests that these proteins are under independent regulatory mechanisms. In previous studies we have found the behavior of these two viral marker proteins to be coordinated, i.e., estrogen administration elicits both or neither, depending upon the mouse strain used.

It is reasonably clear that a lack of estrogen receptors in the uterus cannot explain our results. There is histological evidence of endometrial hyperplasia in the uteri of the "unstimulated" old mice, which implies both an endogenous estrogen source and the presence of receptors. Studies in postmenopausal women suggest that in the absence of functional ovaries, estrogens can arise by peripheral (e.g., adipose tissue) conversion of androgens (10). Others have implicated the adrenals as an estrogen source in women (11) and in mice (12). It has been suggested (13) that the estrogen receptor population degenerates after long-term hormone absence. Eisenfeld and Axelrod (14) demonstrated that pretreatment of ovariectomized rats with estrogen increased the capacity of the uterus to bind estradiol. We found no change in viral-type DNA polymerase in the uterus if the older mice were pretreated for 2 weeks with estrogen. As we have pointed out, the actual hormone-mediated increases in uterine weight, total protein, and p30 were nearly the same in both groups. Expressed on a percentage basis, however, the increases are greater because of the very low baseline values for the young, unstimulated ovariectomized animals. In spite of these indications of uterine growth, the DNA data imply that cellular proliferation in response to estrogen has not occurred in the uteri of the aged mice, whereas there was a threefold increase in DNA per uterus in the young animals upon stimulation.

Gonadectomy has been known for some time to produce adrenal cortical tumors (for review, see Ref. 15). Adrenal cortical tumors might well produce hormones which could block an estrogen effect at the genetic level in derepressing or activating genes and

A

FIG. 2. Longitudinal sections of NIH Swiss mouse uteri stained with hematoxylin and eosin. A. Uterus from young, ovariectomized animal. B. Uterus from young, nonovariectomized mouse. C. Uterus from aged, ovariectomized animal. Arrow points to the wall of an endometrial cyst frequently seen in uteri of aged, ovariectomized mice. All magnifications are $\times 31$.

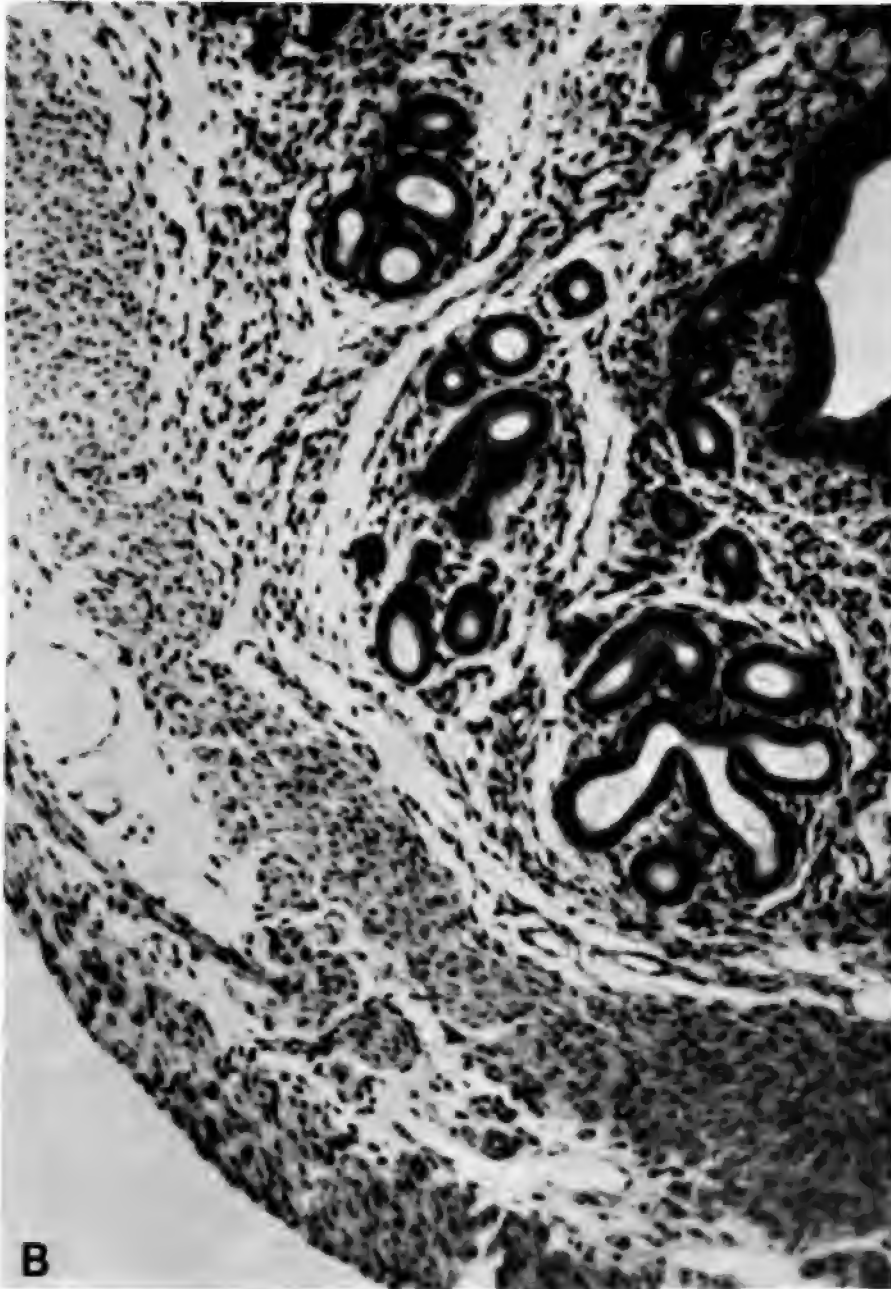


FIG. 2. *Continued.*

A replication while not affecting as much cytoplasmic effects such as membrane permeability and translational steps related by increases in uterine weight and p30 protein. Indeed, we have found in preliminary experiments that cortisol can par-

tially block estrogen-mediated increases in uterine p30 levels in young ovariectomized NIH Swiss mice (data not shown).

Summary. Effects of estrogen administration were compared in young (2–5 months) and aged (2.0–2.5 years), ovariectomized

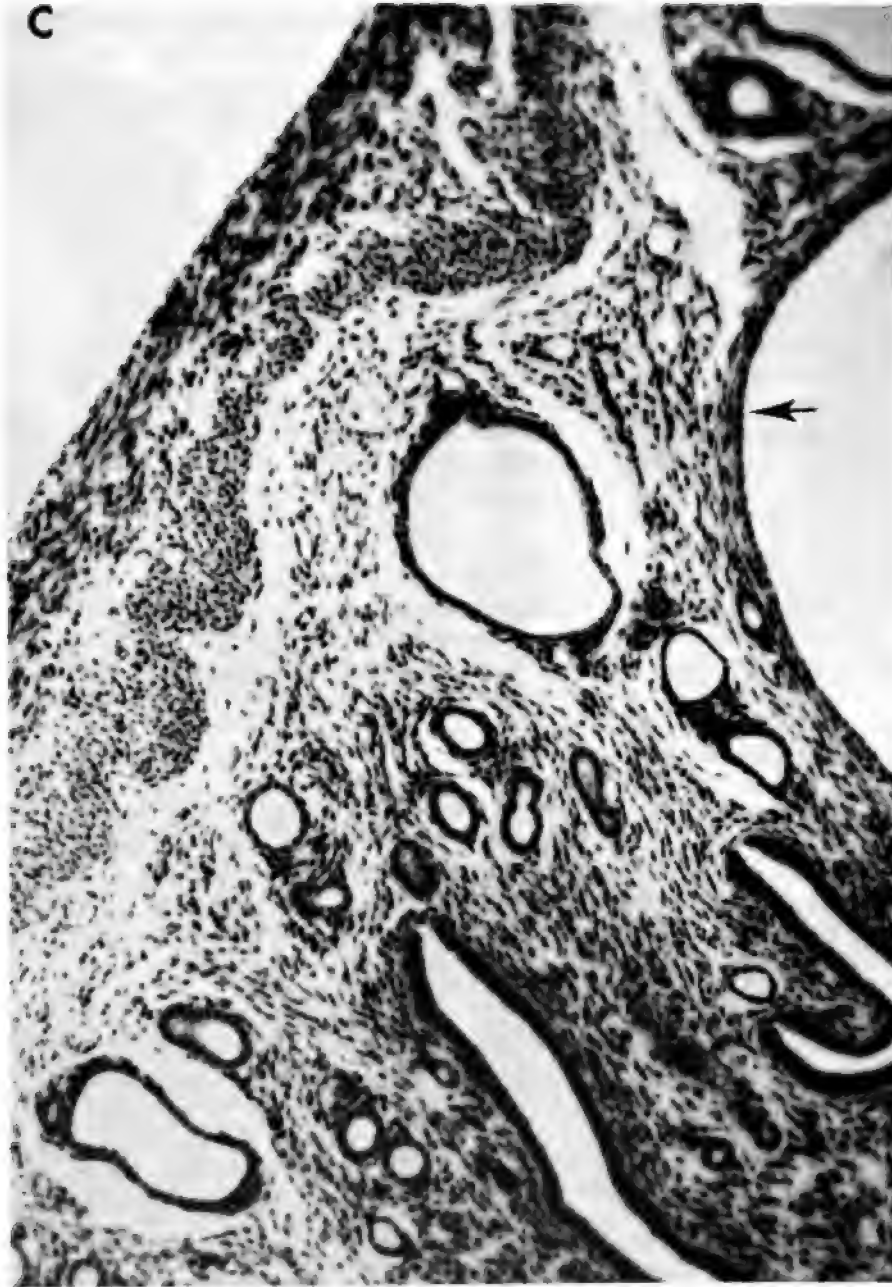


FIG. 2. *Continued.*

NIH Swiss mice. Two murine leukemia virus proteins, p30 and RNA-directed DNA polymerase, were markedly elevated by estrogen in the uteri of young, ovariectomized mice, but behaved differently in aged animals. The polymerase was low or absent in the uteri of aged mice and showed no in-

crease in response to estrogen. In contrast p30 responded to estrogen in the aged animals much the same as in the younger mice. This implies that the production of these viral proteins is under separate control mechanisms.

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Treatment of Cardiac Sensitivity to Hyperkalemia with Aldosterone, Renin, or Insulin¹ (39482)

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In a previous study of transmembrane K⁺ transfer in pancreatectomized (pancx)-nephrectomized (nephx) dogs K⁺-loaded by an infusion of 2 mEq KCl/kg/hr, an unexpected change in the cardiac effect of K⁺ was observed; the cardiotoxic concentration of serum K⁺ (compared to controls) fell by more than 2 mEq/liter to the level previously found in adrenalectomized (adrenx) dogs (1). In the present investigation it was found that treatment with aldosterone, renin, or insulin significantly decreased cardiac sensitivity to K⁺ and restored prelethal serum K levels to the control range.

Methods. Data were compiled from 33 dogs of either sex that weighed between 15.5 and 26.0 kg and were fasted for 18 hr before an experiment. All were anesthetized with 30 mg/kg sodium pentobarbital iv, connected to a Harvard respirator, and infused with about 25 ml/hr of 0.15 M NaCl. Thirty of the animals were bilaterally nephrectomized through a midline abdominal incision. Twenty-seven were pancreatectomized by the avulsion method (2) before loading with K⁺; 12, 2 to 6 days before an experiment (diabetes was controlled with regular insulin until 18 h before infusion was commenced), and 15, immediately after bilateral nephrectomy. Dogs were loaded with K⁺ by discontinuing NaCl infusion and connecting them to a Harvard peristaltic pump that delivered 30 ml/hr of a KCl solution of such concentration that each animal re-

ceived 2 mEq/kg/hr in a cephalic vein infusion, dogs were connected to a Hewlett-Packard ECG machine and monitored at frequent intervals; K⁺ administered until prelethal ECG changes appeared—ventricular bradycardia (less than 40 beats/minute, bizarre QRS, or ventricular flutter).

The dogs were divided into five groups (Table I). Group A, the control group, consisted of nine animals; six were infused with 2 mEq/kg/hr KCl 2 hr after nephrectomy, and three were infused 2 to 6 days after pancreatectomy. Group B comprised 8 nephrectomized dogs; five were pancreatectomized 2 to 6 days before nephrectomy and KCl infusion, while three were pancreatectomized immediately following nephrectomy and K⁺-loaded 70 min later. Serum insulin levels are well below or even unmeasurable (3). Group C, made up of six nephrectomized-pancreatectomized dogs that were treated with infusions of KCl and 3 µg aldosterone/kg/hr (in 30 ml/hr of ~1 ml of a 1% ethanol solution in 240 ml ion-free water; the latter was started in the opposite cephalic vein immediately after nephrectomy (i.e., about 2 hr before KCl infusion) continued for nearly 4 hr, to the end of the experiment. Group D, six pancreatectomized-nephrectomized dogs that were treated with infusions of KCl and 5 µg insulin/kg/hr with 20 µg glucagon (each/kg/hr) in 30 ml/hr of water was started immediately after nephrectomy and continued for about 4 hr to the end of the experiment. When glucose fell below 50 mg/100 ml, doses of a 50% glucose solution in water were injected iv. Group E include

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TABLE I. INFUSED TO PRELETHAL LEVEL WITH 2 mEq KCl/kg/hr.

Group	Total K ⁺ infused (mEq/kg)	Prelethal serum K ⁺ (mEq/liter)
nephx (6) or x (3)	4.2 ± 0.9	9.94 ± 0.16 ^a
nephx-pancx (B)	1.2 ± 0.1	10.01 ± 0.11
nephx-pancx, sterone (C)	2.20 ± 0.06	7.68 ± 0.18
nephx-pancx, lin (D)	2.71 ± 0.06	9.84 ± 0.22
nephx-pancx, 1 (E)	5.4 ± 0.09	<.001
x (12) adrenx)	3.4 ± 0.05	9.26 ± 0.26
	1.89 ± 0.14	<.001
		7.59 ± 0.09
		NS

Number of dogs.

Mean and SEM.

Previously reported (1).

> 0.05 not significant: *t* test.

nephrectomized–nephrectomized dogs in 1 K⁺-loading was accompanied by infusion of enough of a saline solution of hog to raise mean blood pressure by 30 mm Hg; renin infusion was begun immediately after nephrectomy and continued for 4 hr to the end of KCl administration. Numerous blood samples were obtained from an exposed femoral vein immediately after anesthesia, before KCl infusion was begun, at half-hour intervals as it proceeded, when it was discontinued, i.e., when the prelethal ECG changes appeared (“end point”). Serum K⁺ was determined with an Instrumentation Laboratories Flame Photometer using lithium as an internal standard. In the pancreatectomized and nephrectomized–pancreatectomized dogs serum insulin was measured by the method of Morand Lazarow (4), and blood glucose by the glucose oxidase method (5). In four nephrectomized–pancreatectomized dogs arterial blood pH and serum Ca were also measured; the former with Radiometer Base Analyzer, the latter with a Perkin-Elmer Atomic Absorption Flame Photometer.

Results. The dogs pancreatectomized 2 to 6 days before, seemed the same as any other dogs subjected to operation of similar type, but all were frankly diabetic, with blood glucose greater than 250 mg/100

ml, urine glucose 1–2 g/100 ml (Clinitest), no acetonuria (Acetest), and serum insulin <2.5 μU/ml. The dogs pancreatectomized 70 min before were not yet diabetic (6) at the time of KCl infusion, i.e., they had no hyperglycemia, glycosuria, or acetonuria, although insulin levels averaged only approximately one-third normal. In four nephrectomized–pancreatectomized dogs serum Ca and arterial pH were within normal limits. Neither pancreatectomy, nephrectomy, nor pancreatectomy–nephrectomy significantly altered the mean normal (pre-infusion) level of serum K⁺ (4–5 mEq/liter).

Group A (controls), nephrectomized or pancreatectomized and KCl infusion (Table I). The end point was similar in both sets of dogs. The mean serum K concentration at which prelethal ECG changes appeared differed by less than 0.3 mEq/liter from the average (10.2 mEq/liter) determined in eight dogs previously reported (three each of these were normal or bilaterally ureter-ligated, while two were ligated–pancreatectomized animals (7, 8)). In the pancreatectomized dogs serum insulin was always <2.5 μU/ml and blood glucose over 250 mg/100 ml.

Group B, nephrectomized–pancreatectomized, with KCl infusion 70 min to 6 days after pancreatectomy (Table I). In all members of the group there was a highly significant increase of sensitivity to hyperkalemic cardiotoxicity: prelethal ECG changes appeared at a mean serum K concentration ~2.3 mEq/liter below that of dogs that were either pancreatectomized or nephrectomized. The blood glucose response was not uniform—it was consistently >250 mg/100 ml in those with pancreatectomy of 2 to 6 days duration, but within normal limits (90–120 mg/100 ml) in those pancreatectomized 70 min before; however, during KCl infusion serum insulin was <2.5 μU/ml in all specimens from both. Increased sensitivity to K⁺ cardiotoxicity was almost identical in hyperglycemic and normoglycemic dogs. In the four animals in which arterial pH and serum Ca were measured, these were within usual limits during the entire course of KCl infusion; calcium varied between 9.1 and 10.6 mEq/100 ml—pH between 7.24 and 7.43.

Group C, nephrectomized–pancreatecto-

mized, infused with KCl 70 min later and treated with aldosterone (Table I). Aldosterone treatment abolished cardiac sensitivity to hyperkalemia; mean prelethal concentration of serum K^+ rose by almost 2.2 mEq/liter to nearly the same level found in dogs that were either nephrectomized or pancreatectomized. Blood glucose stayed within normal limits but serum insulin was consistently $<2.5 \mu\text{U/ml}$ during KCl administration.

Group D, nephrectomized-pancreatectomized, infused with KCl from 70 min to 6 days after pancreatectomy and treated with insulin and glucagon (Table I). Insulin treatment markedly decreased cardiac sensitivity to hyperkalemia—mean prelethal serum K^+ (as in aldosterone treated dogs) was highly significantly greater than in untreated nephrectomized-pancreatectomized dogs. Serum insulin was not measured; blood glucose tended to fall, but was kept in the normoglycemic range by the injection of two to three, 10 ml doses of a 50% glucose solution. In two nephrectomized-pancreatectomized dogs treated with glucagon alone, there was moderate hyperglycemia and no suggestion of improved cardiac sensitivity to K^+ .

Group E, nephrectomized-pancreatectomized, infused with KCl 70 min later and treated with renin (Table I). Treatment with renin almost completely restored cardiac sensitivity to K^+ . Mean prelethal serum K^+ was only about 0.4 mEq/liter less than in the control animals of Group A. During KCl infusion serum insulin was $<2.5 \mu\text{U/ml}$ and blood glucose between 90 and 120 mg/100 ml in all specimens.

Discussion. In dogs infused with 2 mEq KCl/kg/hr after simultaneous nephrectomy and pancreatectomy, there was a highly significant ($P < 0.001$) increase of cardiac sensitivity to elevated serum K^+ . Since neither nephrectomy nor pancreatectomy alone produced any substantial change of cardiac sensitivity to hyperkalemia, it was entirely unclear why sensitivity should be so remarkably augmented when the operations were combined. The only clue was the previous finding that dogs infused with 2 mEq KCl/kg/hr 4 hr after bilateral adrenalectomy (with and without nephrectomy) had an al-

most identical increase of cardiac sensitivity to hyperkalemia (1, Table I). Functional adrenalectomy due to adrenal exhaustion was considered, but the scope and duration (<1 h) of the operative procedure made it unlikely. The usual serum Ca and arterial blood pH levels (in those nephrectomized-pancreatectomized dogs in which they were measured) ruled these out as causes of increased cardiac sensitivity.

Although there is no obvious connection between adrenalectomized dogs and those with simultaneous pancreatectomy and nephrectomy, the similar cardiac responses to hyperkalemia suggested a possible relation. In work to be published we found that cardiac sensitivity to K^+ returns to the normal range in K^+ -loaded adrenalectomized dogs that are nephrectomized and treated with 3 μg aldosterone/kg/hr. The same treatment proved effective in nephrectomized-pancreatectomized animals—an infusion of 3 μg aldosterone/kg/hr, started before and continued during K^+ loading, consistently restored prelethal serum K^+ to control levels (Table I). This observation suggested that nephrectomized-adrenalectomized and nephrectomized-pancreatectomized dogs were equivalent preparations in which KCl failed to provoke aldosterone secretion; in the former because of actual absence of the adrenals, in the latter possibly because of functional inability to stimulate secretion of the hormone—thus making them both aldosterone deficient.

There is evidence that mineralocorticosteroids are involved in maintenance of the normal resting membrane potential of myocardial fibers in rats, and of their sensitivity to K^+ in dogs. Following adrenalectomy both are diminished, and each is improved by treatment with mineralocorticoids (1, 9, see above).

K^+ salt infusion acts on the adrenal to stimulate aldosterone secretion in intact and nephrectomized dogs (10). In these and in pancreatectomized animals (absence of insulin alone does not influence K^+ cardiotoxicity (Table 1)) normal cardiac sensitivity to K^+ suggests adequate aldosterone secretion in response to KCl infusion. However, in nephrectomized-pancreatectomized dogs aldosterone secretion seems inadequate—there

ly significant diminution of cardiac sensitivity to K⁺ unless exogenous hormone is administered or the animals are treated with insulin. While the cardioprotective effect of the former may stem from its ability to stimulate aldosterone secretion, the latter with no such property may exert its protective effect in nephrectomized-pancreatectomized dogs by mediating the stimulation of aldosterone secretion by KCl. In nephrectomized animals become hypokalemic in about 30 min (10)). K⁺ salts stimulate aldosterone secretion, apparently by transfer of K⁺ to intracellular fluid (ICF) in the glomerulosa (11) and insulin has the ability to transfer infused K⁺ to the cells (12). Endogenous insulin stimulated by angiotensin (7) may perform the same office in nephrectomized dogs. At any rate, as has been noted clinically that elevation of serum K⁺ may be associated with hyperkalemia in hyporeninemic diabetes (13).

On the other hand, all of our results can be adequately explained if nephrectomy-pancreatectomy reduces cardiac sensitivity to K⁺ by inactivation of a sensitizing substance which is in turn inactivated by infusion of aldosterone, insulin or renin. Furthermore, our observations may stem from local effects of insulin (13) or angiotensin (14). However, the validity of this explanation is rather impaired by our finding that treatment with insulin or renin is ineffective in adrenalectomized-pancreatectomized dogs—dogs in which cardiac sensitivity to K⁺ is completely restored when treated with aldosterone (to be published).

These are centers (not our own at present) where numbers of hyporeninemic diabetes with low aldosterone levels are well known. In these, it may be possible to demonstrate the role of insulin in the response of the heart to KCl infusion. The results of our study have considerable value clinically—presently there are many anephric diabetes which are prone to cardiac complications (15).

Infusion of KCl into a dog not only serves to maintain K⁺, but it also provides for urinary excretion (pancreatectomized dogs of 1963) and transmembrane K⁺ transfer.

In the present experiment there is no discernible relation between total K⁺ infused and prelethal serum K⁺ concentration (Table I).

Cross circulation experiments should help clarify the mechanism of increased cardiac sensitivity to K⁺ while assay of serum aldosterone should establish the importance of insulin in mediating stimulation of aldosterone secretion by KCl infusion, in nephrectomized-pancreatectomized dogs.

Summary. In pancreatectomized or nephrectomized dogs K⁺-loading by infusion of 2 mEq KCl/kg/hr produces ECG evidence of prelethal hyperkalemic cardiotoxicity when serum K⁺ reaches about 10 mEq/liter. If, however, the operations are combined and the KCl infused into nephrectomized-pancreatectomized animals, there is a highly significant increase in cardiac sensitivity to hyperkalemia; prelethal ECG changes appear when mean serum K⁺ is ~7.7 mEq/liter ($P < 0.001$), a level almost identical with that found in dogs loaded with K⁺ 4 hr after bilateral adrenalectomy. Treatment of nephrectomized-pancreatectomized dogs with aldosterone, renin, or insulin decreases cardiac sensitivity to K⁺ and raises the prelethal cardiotoxic level of serum K⁺ to the range found in controls. The results suggest the possibility of a role for insulin in stimulation of aldosterone secretion by KCl infusion.

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Role of the Thyroid Gland to the Effects of Estradiol on Adrenal Corticosterone Secretion in Rats (39483)

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Previous studies have demonstrated that estradiol affects various components of the hypothalamic-pituitary-adrenocortical axis in rats (1). The net effect of these actions is enhancement of adrenocortical secretion. Ovariectomy produces a fall in corticosterone secretion, an effect reversed by estradiol replacement. The decline in corticosterone output after ovariectomy is greater than can be explained by changes in plasma ACTH levels (2-4). The apparent discrepancy between ACTH and corticosterone output after adrenalectomy is attributable to changes in intra-adrenal metabolism or corticosterone release by castrates (4, 5). In the absence of androgens, adrenal 5α -reductase activity is increased, resulting in the intra-adrenal conversion of corticosterone (B) to 5α -dihydrocorticosterone (DHB) and $3\beta,5\alpha$ -tetrahydrocorticosterone (THB). Secretion of these compounds accounts in part for the increase in corticosterone. Administration of estradiol to castrates lowers reductase activity and restores corticosterone secretion to control levels (4).

Kitay *et al.* (6) have proposed that the effects of estradiol on corticosterone secretion in rats are mediated by the thyroid gland. These investigators have shown that estradiol stimulates pituitary TSH secretion, thereby increasing thyroid activity, which in turn increases adrenal secretion. Previous studies had demonstrated an increase in adrenal steroid output after thyroid hormone administration. In addition, the effects of estradiol on adrenal function appear to be diminished in thyroidectomized rats. However, indirect methods of evaluation of adrenal secretion were used and a physiologic role for estradiol in male animals seems unlikely. Further, since estradiol acts on several components of the pituitary-adrenal regulatory system (1), it remains to be established

whether its effects on adrenal secretion are fully or only in part mediated by the thyroid gland. For these reasons, further studies were conducted to determine the relation of the thyroid gland to estrogenic effects on adrenocortical secretion.

Materials and Methods. Female rats of the Sherman strain, obtained from Camm Research Institute, Wayne, New Jersey, were used in all experiments. Animals were maintained under standardized conditions of light (0600-1800) and temperature ($22.0 \pm 0.5^\circ\text{C}$) on diet consisting of Purina Laboratory Chow and water *ad lib*. Thyroidectomies, when indicated, were performed by the breeder on animals 25-30 days old. Ovariectomies were performed 1 week later with no further manipulation for 4 weeks. At that time a single subcutaneous injection of estradiol cyclopentyl-propionate (Estradiol Cypionate, Upjohn) $50 \mu\text{g}/100 \text{ g bw}$ was administered. Four weeks later, adrenal vein blood was collected from each rat (7) following induction of anesthesia with sodium pentobarbital ($4.5 \text{ mg}/100 \text{ g body wt}$). Plasma was separated immediately and frozen for subsequent analysis. Corticosterone $1,2\text{-}^3\text{H}$ ($\sim 10^{-3} \mu\text{Ci}$, SA 40 Ci/mmol) was added to each sample for subsequent recovery calculations. Chloroform extracts were chromatographed on a Bush B1 system (8) and the zones corresponding to those of authentic corticosterone, 5α -dihydrocorticosterone (DHB) and $3\beta,5\alpha$ -tetrahydrocorticosterone (THB) were eluted with methanol. Identification and homogeneity of these products has been established previously (4) by paper and thin-layer chromatography, infrared spectroscopy, and mass spectrometry. All three steroids were quantitated using a competitive protein binding technique (4, 9) with the appropriate standards. Reference steroids were obtained from Ika-pharm, Ramat-Gan, Israel. The sensitivity

of the assay as employed was 0.5 ng for B, 5 ng for DHB, and 10 ng for THB. The differences in sensitivity reflect the relative affinities of each compound for canine plasma binding protein. Aliquot sizes were adjusted to accommodate the various standard curves. A portion of each corticosterone eluate was counted for ^3H content on a Packard Tri-Carb liquid scintillation spectrometer to determine steroid recoveries. The recoveries of corticosterone, DHB and THB in each sample were essentially identical and averaged about 70%. Adrenal 5α -reductase activity and corticosterone production by adrenal homogenates were assayed as previously described (5). Corticosterone was determined fluorometrically (10).

Results. As previously noted, estradiol administration to ovariectomized rats increased adrenal weight (Table I), presumably as a result of its effects on ACTH secretion (2). Removal of the thyroid gland markedly reduced adrenal weight and prevented the stimulatory response to estradiol. Corticosterone production by adrenal homogenates *in vitro* was increased equally by estradiol administration to castrated rats *in vivo* in the presence or absence of the thyroid gland. Similarly, the inhibitory effect of estradiol on adrenal 5α -reductase activity did not require the presence of the

thyroid gland. Since we had previously demonstrated (4) that the effects of castration and gonadal hormone replacement on corticosterone secretion were mediated in part by changes in adrenal reductase activity, these observations suggested that the thyroid was not required for estrogenic stimulation of corticosterone output. Direct confirmation was obtained by evaluation of corticosterone and its 5α -reduced metabolites in adrenal venous blood.

Estradiol administration to ovariectomized rats increased *in vivo* corticosterone secretion either in the presence or absence of the thyroid gland (Table I). However, the increment was substantially smaller ($P < 0.01$) in thyroidectomized animals. In castrates with intact thyroids, the increase in corticosterone secretion produced by estradiol exceeded the decline in 5α -dihydrocorticosterone (DHB) and $3\beta,5\alpha$ -tetrahydrocorticosterone (THB) output. As a result, total (corticosterone + DHB + THB) steroid secretion was significantly increased by estradiol.

In thyroidectomized castrates, on the other hand, the decline in DHB and THB secretion after estradiol treatment corresponded in magnitude to the increase in corticosterone. Thus, total (corticosterone + DHB + THB) steroid secretion, like adrenal weight, was unaffected by estradiol in

TABLE I. EFFECTS OF ESTRADIOL ADMINISTRATION ON ADRENAL REDUCTASE ACTIVITY AND STEROID SECRETION IN OVARIECTOMIZED AND THYROIDECTOMIZED FEMALE RATS.^a

Group	Ovariectomized	Ovariectomized + estradiol	Ovariectomized + thyroidectomized	Ovariectomized + thyroidectomized + estradiol
Body weight (g)	285 ± 11	258 ± 7 ^b	193 ± 13 ^b	174 ± 9
Adrenal weight (mg)	72.2 ± 3.6	81.7 ± 3.0 ^b	41.6 ± 4.8 ^b	36.3 ± 3.6
Corticosterone production <i>In Vitro</i> (μg/100 mg/30 min)	12.6 ± 2.0	20.8 ± 2.6 ^b	10.8 ± 1.2	21.8 ± 2.9 ^c
Reductase activity (μg B reduced/10 mg/hr)	7.7 ± 2.0	0.4 ± 0.2 ^b	8.7 ± 1.6	0.5 ± 0.2 ^c
Steroid secretion <i>in vivo</i> (μg/kg/hr)				
Corticosterone (B)	72.3 ± 8.9	148.7 ± 12.9 ^b	54.9 ± 6.3	86.3 ± 7.2 ^c
DHB ^d	31.8 ± 3.6	12.3 ± 2.2 ^b	20.1 ± 3.9 ^b	4.9 ± 1.1 ^c
THB ^e	21.4 ± 2.9	8.9 ± 1.9 ^b	12.9 ± 2.9 ^b	2.2 ± 0.7 ^c
Total (B + DHB + THB)	125.6 ± 12.8	169.9 ± 13.8 ^b	87.9 ± 8.9 ^b	93.4 ± 8.8

^a Values expressed as mean ± SE; 8–10 rats per group.

^b $P < 0.05$ (vs ovariectomized group).

^c $P < 0.05$ (vs ovariectomized + thyroidectomized group).

^d 5α -dihydrocorticosterone.

^e $3\beta, 5\alpha$ -tetrahydrocorticosterone.

thyroidectomized castrates, indicating little change in ACTH secretion.

Discussion. Labrie, Fortier, and co-workers (5) have proposed that estradiol produces an increase in adrenal steroid output in rats by increasing thyroid hormone secretion, which in turn increases corticosteroid binding protein activity in plasma, thereby diminishing plasma "free" corticosteroid levels and stimulating ACTH secretion. Our study supports, only in part, the conclusions of Labrie *et al.* (6). When given to ovariectomized rats with intact thyroid glands, estradiol increases corticosterone secretion both by increasing tropic hormone (ACTH) stimulation of the adrenal cortex (2) and by increasing the intra-adrenal conversion of corticosterone to 5α -dihydrocorticosterone (DHB) and $3\beta,5\alpha$ -tetrahydrocorticosterone (THB) (4). The increase in plasma ACTH previously shown to be produced by estradiol administration to castrates (2), manifested in the present studies as increases in adrenal size and in total secretion of corticosterone plus its principal adrenal metabolites (DHB + THB). Since neither these changes was seen after estradiol administration to rats that were thyroidectomized as well as ovariectomized, the effects of estradiol (on adrenal secretion) resulting in changes in ACTH secretion do appear thyroid-mediated. However, estradiol, like corticosterone (13) exerts direct effects on intra-adrenal steroid metabolism. Inhibition of adrenal 5α -reductase activity by estradiol is independent of the thyroid gland. Estradiol administration to thyroidectomized-castrated rats decreases the production and secretion of DHB and THB, thereby preserving corticosterone without affecting total steroidogenesis. Consequently, estradiol increases adrenal corticosterone secretion in ovariectomized rats in the absence of the thyroid gland by changing the composition rather than quantity of steroid secreted. Thus, studies dealing with gonadal hormone effects on pituitary-adrenocortical function must take account the multiple sites of action of estrogen and androgen. In addition, the results further emphasize the importance of thyroid-adrenal interactions in the overall regulation of adrenal steroid secretion.

Summary. The relation of the thyroid

gland to estrogenic effects on adrenal corticosterone secretion was examined in ovariectomized rats. Estradiol administration to castrates increased corticosterone output by increasing ACTH secretion and by decreasing adrenal 5α -reductase activity, diminishing the intra-adrenal conversion of corticosterone to 5α -reduced metabolites. When given to rats that were thyroidectomized as well as castrated, estradiol produced a far smaller increase in corticosterone secretion. The increment in corticosterone obtained in thyroidectomized rats was fully accounted for by the effects of estradiol on adrenal 5α -reductase activity. The results indicate that effects of estradiol on adrenal secretion resulting secondarily from changes in ACTH secretion are thyroid-dependent, whereas direct effects on intraadrenal steroid reductive pathways are not.

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Elevated Cyclic GMP Levels in Rabbit Acetylcholine Tre:

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At the present time, the roles of cyclic nucleotides in the regulation of cardiac contraction are under active investigation. Most of this work is centered upon the connection between catecholamine stimulation and cardiac levels of cyclic 3',5' adenosine monophosphate (cAMP). Thus, epinephrine has been shown to increase the levels of cAMP in various heart tissues concomitant with increases in either rate or force of contraction (1-4). Also, cAMP and its dibutyryl derivatives have both been demonstrated to increase rate and/or force of contraction in various isolated cardiac tissue preparations (5, 6).

Comparatively little work has been performed on the elucidation of a possible role for cyclic 3',5' guanosine monophosphate (cGMP) in the regulation of the mammalian heart. Several years ago George *et al.* (7) reported that acetylcholine caused a significant rise in cGMP content in isolated perfused spontaneously beating rat hearts, while cAMP content either did not change or decreased slightly. In these studies there was a strong inverse correlation between cGMP content and tension development in the whole rat heart. More recently this work has been further amplified (8) to show that the inverse correlation between cGMP levels and tension development in the isolated perfused rat heart given acetylcholine does not depend upon changes in heart rate, and that the effect of acetylcholine on cGMP levels can be abolished by pretreatment with atropine.

Since the cholinergic innervation of the

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concentration of $5 \times 10^{-7} M$ (calculated salt) was added alone or in combination with atropine sulfate at a concentration of $10^{-7} M$ and at various intervals the hearts were frozen in Wollenberger clamps and had been precooled in liquid nitrogen. AMP and cGMP content of the tissue was then assayed by a protein binding technique (11) and radioimmunoassay (12), respectively.

Isolated rabbit atrium/vagus nerve preparation. In this series of experiments, New Zealand white rabbits weighing between 2–3 kg were anesthetized with sodium pentobarbital (50 mg/kg, iv) and their chests were opened through a mid-sternal incision. Respiration was maintained by applying positive pressure ventilation through a tracheal cannula, such that a constant minute volume of approximately 1 liter was maintained. Following excision of the pericardium, a single suture was passed through the apex of the right atrial appendage, to facilitate rapid removal of the atrium. Heart rate was determined from a standard electrocardiogram taken from needle electrodes inserted subcutaneously. The right cervical vagus was isolated, crushed centrally, and connected across shielded bipolar electrodes for stimulation (physiograph model SI-10 stimulator, Palmer shielded electrodes). The distribution of the right vagus in the chest is known to be localized almost exclusively to the sino-atrial node and the right atrium (13). In all experiments, test stimulation was carried out to determine stimulus intensities which would yield approximately a 50% slowing of heart rate from control. Then, following a 10 min rest pe-

riod, vagal stimulation was reapplied for various time intervals. Then, the right atrium was rapidly excised and frozen in Wollenberger clamps precooled in liquid nitrogen. Control hearts were treated identically except for the final vagal stimulation. In some experiments atropine sulfate, 2 mg/kg, iv, was administered to the rabbit immediately following the test stimulation, and the experiment was completed as before. Cyclic nucleotide content of the atrial tissue was assayed as previously described (11, 12).

Statistical analysis. Differences in group means were determined using the appropriate "t" test (14). Regression lines were fitted by means of the method of least squares. Correlation coefficients and the significance of regressions were calculated using standard regression analyses (15).

Results. Isolated rabbit atrial preparation. Table I illustrates the changes in atrial cGMP levels, cAMP levels, and force of contraction in isolated paced rabbit atria following the addition of acetylcholine ($5 \times 10^{-7} M$). As can clearly be seen, the addition of acetylcholine to the tissue bath resulted in increases in atrial cGMP levels from a control value of 92 ± 8 nmole/kg to 129 ± 14 nmole/kg at 5 sec, 195 ± 29 nmole/kg at 10 sec, and 166 ± 18 nmole/kg at 15 sec following drug addition. All values were significantly different from control. At none of these time intervals was the atrial level of cAMP altered significantly ($P > 0.05$) from the control value of 715 ± 50 nmoles/kg. It is of importance here to note that although acetylcholine caused a significant ($P < 0.05$) decrease in the force of

TABLE I. EFFECTS OF ACETYLCHOLINE AND ATROPINE ON RATE, FORCE OF CONTRACTION, AND CYCLIC NUCLEOTIDE CONTENT IN ISOLATED PACED RABBIT ATRIA.

Experimental conditions	n	cGMP (nmole/kg tissue)	cAMP (nmole/kg tissue)	Heart rate (% control)	Isometric tension (% control)
Control	5	92 ± 8	715 ± 50	100	100 ± 3
Acetylcholine ^a 5 sec	5	129 ± 14^c	682 ± 88	100	97 ± 3
Acetylcholine 10 sec	5	195 ± 29^c	642 ± 55	100	90 ± 2^c
Acetylcholine 15 sec	5	166 ± 18^c	618 ± 49	100	84 ± 5^c
Atropine ^b	5	84 ± 11	687 ± 41	100	101 ± 3
Atropine + acetylcholine ^a	5	96 ± 12	702 ± 23	100	96 ± 3

^a Acetylcholine concentration = $5 \times 10^{-7} M$.

^b Atropine sulfate concentration = $1 \times 10^{-7} M$.

^c Significantly different from control values ($P < .05$).

atrial contraction at both 10 and 15 sec following its addition to the muscle bath, a significant rise in the atrial levels of cGMP preceded the decrease in force of contraction by approximately 5 sec.

Table I also shows the effects of atropine (1×10^{-7} M) on changes in atrial force of contraction and cyclic nucleotide content produced by acetylcholine (5×10^{-7} M). Atropine alone caused no significant ($P < 0.05$) alteration in either force of contraction or cyclic nucleotide content of the isolated atria. However, pretreatment of the tissue with atropine successfully attenuated both the decrease in force of contraction and the rise in atrial cGMP content brought about by addition of acetylcholine to the muscle bath.

In situ rabbit atrium/vagus nerve preparation. Table II illustrates the effect of vagus nerve stimulation on right atrial cyclic nucleotide content and heart rate. The atrial levels of cGMP (expressed as percentage of control) were significantly ($P < 0.05$) elevated at 3 sec ($125 \pm 7\%$), 5 sec ($135 \pm 15\%$), and 15 sec ($175 \pm 37\%$) following the onset of vagal stimulation, but had returned to near control levels ($105 \pm 6\%$) at the end of 30 sec of stimulation, even in the face of a continued slowing of heart rate. At no time period studied was the atrial content of cAMP found to be significantly ($P > 0.05$) different from that of the control tissues.

Table III shows the effects of pretreatment with atropine (2 mg/kg, iv) on the ability of vagal nerve stimulation to alter heart rate and atrial cGMP content. Atropine pre-treatment was observed to prevent both the decrease in heart rate induced by vagal stimulation as well as the increase in

TABLE III. EFFECTS OF VAGAL NERVE STIMULATION ON RABBIT RIGHT ATRIAL CYCLIC GMP CONTENT AND HEART RATE FOLLOWING ATROPINE PRETREATMENT.

Experimental condition	n	Rate (% control)	cGMP (% control)
Atropine ^a	4	100 \pm 4	100 \pm 7
Atropine ^a + vagal stimulation (15 sec)	4	93 \pm 2	89 \pm 7

^a Atropine pretreatment = 2 mg/kg atropine sulfate, iv.

atrial cGMP content associated with this stimulus.

In an attempt to assign a functional role to the alteration in atrial cGMP content observed following cholinergic stimulation, cGMP changes were plotted against either changes in the rate of the *in situ* rabbit atrium during vagal stimulation (Fig. 1), or against the changes in force of contraction of the isolated paced rabbit atrium following addition of acetylcholine (Fig. 2). The results of these plots indicate no correlation between changes in atrial cGMP content and changes in atrial rate produced by vagal stimulation ($r = -0.26$), but a good correlation between alterations in atrial cGMP content and changes in force of contraction brought about by administration of acetylcholine to isolated paced rabbit atria ($r = 0.75$).

Discussion. Results of the present study demonstrate that muscarinic cholinergic stimulation of rabbit atrial muscle, both *in vitro* and *in vivo*, causes an increase in atrial content of cGMP which occurs with, or precedes the mechanical change produced by such stimulation. In addition, the data show that cholinergic stimulation of the rabbit atrium does not significantly alter the atrial level of cAMP. These findings are in agreement with earlier studies involving the isolated whole rat heart (7). However, it is difficult based on current evidence to assign a definite role to cGMP in the regulation of atrial performance. In our studies, we could find no correlation between the increase in atrial cGMP level produced by vagal stimulation and the concomitant decrease in atrial rate of contraction. This result is in disagreement with previous findings (8) utilizing the isolated spontaneously beating rat heart given acetylcholine, where whole heart

TABLE II. EFFECTS OF VAGAL NERVE STIMULATION ON RABBIT RIGHT ATRIAL CYCLIC NUCLEOTIDE CONTENT AND HEART RATE.

Time (sec)	n	Rate (% control)	cGMP (% control)	cAMP
0	9	100 \pm 5	100 \pm 4	100 \pm 3
3	6	62 \pm 8*	125 \pm 7*	95 \pm 4
5	6	62 \pm 11*	135 \pm 15*	90 \pm 7
15	7	52 \pm 6*	175 \pm 37*	96 \pm 5
30	7	46 \pm 5*	105 \pm 6	102 \pm 2

* Significantly different from control values ($P < .05$).

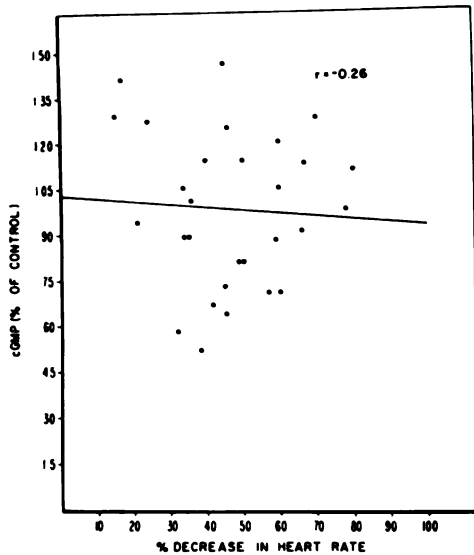


FIG. 1. Relationship between heart rate during vagal stimulation and atrial cGMP content in the *in situ* rabbit atrium. A correlation coefficient of -0.26 was obtained.

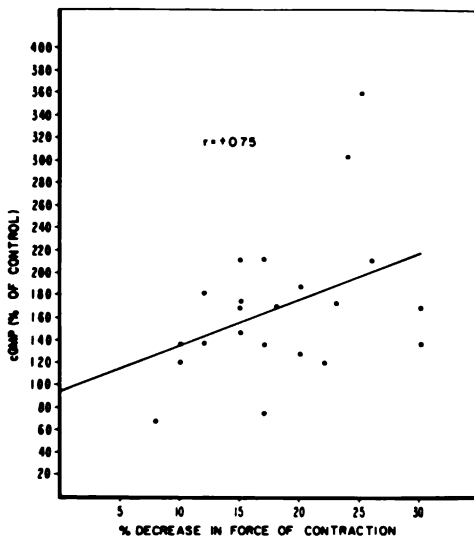


FIG. 2. Relationship between force of contraction and atrial cGMP content in the isolated paced rabbit atrium following addition of acetylcholine ($5 \times 10^{-7}M$). A correlation coefficient of 0.75 was obtained.

cGMP content showed a strong inverse correlation with heart rate. Obviously, nonuniform changes in cyclic nucleotide level within the heart (i.e., atrium vs ventricle, or myocardium vs nodal tissue) could account for the observations, but as yet no such differences have been reported.

In contrast, studies with several different cardiac preparations have shown a decreased inotropic effect associated with an increased tissue content of cGMP (16, 17). In the latter studies (17), increases in myocardial cGMP content preceded the effects of cholinergic stimulation, similar to the results obtained with the atrial preparations of the present report (Tables I and II). Additionally, exogenous cGMP and its derivatives have been demonstrated to decrease force of contraction in preparations of isolated atria (18). Although force of contraction was not determined in our *in situ* rabbit atrium/vagus nerve preparation, the isolated rabbit atria did show a correlation between tissue cGMP content and the decreased isometric force development obtained upon addition of acetylcholine. Even though further *in vivo* studies are required, it seems likely that cGMP may play an important role in the intracellular regulation of cardiac contractility. The mechanism whereby cGMP may exert an effect on cardiac contraction is unclear at present, but interactions with the cAMP system (19) or alterations in calcium fluxes (8) have been previously suggested.

These data provide the first evidence that cardiac cyclic nucleotide levels are subject to alteration through physiological (vagal activation) as well as pharmacological (acetylcholine) intervention.

Summary. In studies designed to investigate the effects of cholinergic stimuli on myocardial cyclic nucleotide content and myocardial performance, an isolated paced rabbit atrial preparation and an *in situ* rabbit right atrium/vagus nerve preparation were utilized. Following vagal stimulation or acetylcholine treatment, increases in atrial cGMP content preceded or occurred at the same time as the mechanical changes associated with cholinergic stimulation. Atrial cAMP content was not affected by these stimuli. Increased cGMP levels were correlated with the decreased force of contraction, but not the decreased chronotropic effects of cholinergic stimulation. These data support the concept that modifications in cardiac performance may be mediated by cGMP. In particular, cGMP may mediate the pharmacological effects of acetylcholine

as well as the physiological effects of vagal stimulation.

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Heterogeneous Nucleation with Urate, Calcium Phosphate and Calcium Oxalate (39485)

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Renal stones frequently occur as mixtures of two or more mineral phases (1, 2). It has been suggested that the formation of such stones is initiated by heterogeneous nucleation (3-5). This process allows the solid nidus to cause "overgrowth" of crystals of different chemical composition (6). Several forms of heterogeneous nucleation have been reported. Brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$), the predominant crystal formed in urine upon induction by (7) or by organic matrix (8) at pH 7, facilitated the nucleation of calcium oxalate (9). Meyer *et al.* (5) have shown that both hydroxyapatite and brushite are capable of inducing heterogeneous nucleation of Ca oxalate. Seeds of monosodium urate monohydrate have been shown to induce heterogeneous nucleation of both calcium phosphate and Ca oxalate (3, 4). This process could account for the formation of containing renal stones in patients with uricosuria, who present no abnormality of Ca metabolism (10).

The following studies will examine on a biochemical basis the heterogeneous nucleation which occurs with Ca oxalate monohydrate, uric acid, monosodium urate monohydrate, brushite, and hydroxyapatite.

Materials and methods. The general procedure for the examination of heterogeneous nucleation is as follows. The precipitant (or "seed" of the solid phase) was added to a solution which was metastably saturated (9) with respect to the precipitate. Without seeding, such a solution does not allow spontaneous precipitation during the period of the study. The formation of precipitate was assessed from the

change in filtrate concentration of the constituent ions.

The ambient solution was obtained by filtration through 0.05- μm Millipore filters. The following seeds were utilized: brushite (Mallinkrodt), hydroxyapatite (L-apatite) (11), Ca oxalate monohydrate (12), uric acid (Sigma) (3), and monosodium urate monohydrate. Monosodium urate monohydrate was prepared as follows. Ten grams of uric acid were dissolved in 2 liters of hot, boiling 0.031 N NaOH solution; pH was kept at 7.2 by titration with HCl or NaOH. The precipitate, which formed upon cooling to room temperature, was "aged" in the same medium at 6°C for 1 week. It was separated by filtration through 0.45- μm Millipore filter, washed with distilled water, and dried at 80°C for 1 day. Direct chemical and X-ray diffraction analysis disclosed pure preparation of monosodium urate monohydrate. The crystal consisted mostly of needles, 8-12 μm in length.

The studies were performed at 37°C under constant stirring with Teflon-coated magnetic bars with the use of freshly-cleaned, new Pyrex glassware (7) at several pHs. Each experiment was run two or three times; results were acceptable only if replicate values agreed within 5%. In the figures presented here, each point represents the mean of replicate experiments.

Heterogeneous nucleation of urate. It was tested for seeds of brushite, hydroxyapatite, and Ca oxalate monohydrate at pH 5.3, 5.7, and 6.7. The test solution at pH 5.3 contained 150 mmole sodium (Na), 50 mmole acetate and 154 mg uric acid/liter. The solution at pH 5.7 had 150 mmole Na, 50 mmole acetate and 360 mg uric acid/liter. The solution at pH 6.7 contained 150 mmole Na, 5 mmole cacodylate and 2560 mg uric acid/liter. These solutions were approximately twofold saturated with respect

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to uric acid, since their uric acid concentrations were approximately twice the solubility of uric acid, measured 2 hr after incubation in similar aqueous solutions (13). Heterogeneous nucleation was induced by adding to each solution seeds of brushite, hydroxyapatite or Ca oxalate monohydrate at a solid:solution ratio of 2:1 (mg:ml). The filtrates, obtained at various times after seeding for 2 hr, were assayed for uric acid. The heterogeneous nucleation of uric acid or urate was shown by the fractional change in the filtrate concentration of uric acid.

After completion of the study, the precipitates were collected by filtration in 0.05- μ m Millipore filters, and washed in distilled water. They were treated in hot, saturated solution of Li_2CO_3 and assayed for Na and uric acid.

Heterogeneous nucleation of Ca phosphate. It was tested for seeds of uric acid, monosodium urate monohydrate, and Ca oxalate monohydrate at pH 5.3, 5.7, 6.7, and 7.4. The solution at pH 5.3 contained 150 mmole Na, 18.9 mmole Ca, and 9.7 mmole phosphorus (P)/liter. The solution at pH 5.7 had 150 mmole Na, 10 mmole Ca, and 9.4 mmole P/liter. The solution at pH 6.7 was 150 mM, 1.35 mM, and 10 mM with respect to Na, Ca and P, respectively. The solution at pH 7.4 contained 150 mmole Na, 0.92 mmole Ca, and 5 mmole P/liter. These solutions were approximately 3.5-fold (7) saturated with respect to brushite. The solid:solution ratio of uric acid seed was 5:1 (mg/ml); that for monosodium urate monohydrate and Ca oxalate was 2:1. Heterogeneous nucleation of Ca phosphate by seeds of uric acid and monosodium urate was examined from the fractional change in the filtrate concentration of Ca over 6–7 hr following seeding. Because the seed of Ca oxalate could undergo partial dissolution, the heterogeneous nucleation of Ca phosphate by Ca oxalate was monitored by the fractional change in the concentration product of Ca and P of the filtrate. Under the conditions of the experiment, the filtrate concentration of Ca did not increase after seeding.

After the completion of the study, the precipitates were treated in 6 N HCl solution and assayed for Ca and P.

Heterogeneous nucleation of Ca oxalate. It was tested for seeds of uric acid, monosodium urate monohydrate and hydroxyapatite at pH 5.3, 5.7, 6.7, and 7.4. The solid:solution ratio for uric acid was 5 mg/ml; that for monosodium urate monohydrate and hydroxyapatite was 2 mg/ml. The test solutions for studies with seeds of uric acid or monosodium urate monohydrate contained 150 mmole Na, 5 mmole cacodylate, 0.4 mmole Ca, and 0.4 mmole oxalate (Ox)/liter. It was approximately sixfold saturated with respect to Ca oxalate monohydrate (12). Heterogeneous nucleation of Ca oxalate was measured from the fractional change in the filtrate concentration of oxalate.

Since hydroxyapatite seed could undergo significant dissolution, the following precautions were undertaken. An excess of hydroxyapatite was incubated to steady-state in aqueous solutions containing 150 mmole Na and 5 mmole cacodylate at pH 5.3, 5.7, 6.7, and 7.4. Appropriate amounts of oxalate as oxalic acid were then added to the filtrates. The solutions were therefore saturated with respect to hydroxyapatite and metastably supersaturated with respect to Ca oxalate. The test solutions at pH 5.3 and 5.7 contained 1.46 mmole of Ca and 0.13 mmole oxalate/liter. The solution at pH 6.7 was 0.28 mM and 0.43 mM with respect to Ca and oxalate, respectively. The solution at pH 7.4 had 0.14 mmole Ca and 2 mmole oxalate/liter. These solutions were approximately six-, six-, five-, and ninefold saturated with respect to Ca oxalate. The heterogeneous nucleation of Ca oxalate by hydroxyapatite was examined from the fractional change in the filtrate concentration product of Ca and oxalate.

The precipitates obtained after the conclusion of the study were treated with 6 N HCl solution, and assayed for Ca and oxalate.

Analytical methods. Uric acid was determined by the uricase method (14), Ca by atomic absorption spectrophotometry, P by the method of Fiske and SubbaRow (15), and oxalate by the technique of Hodgkinson and Williams (16).

Results. Heterogeneous nucleation of urate (Fig. 1). Without seeding, the filtrate con-

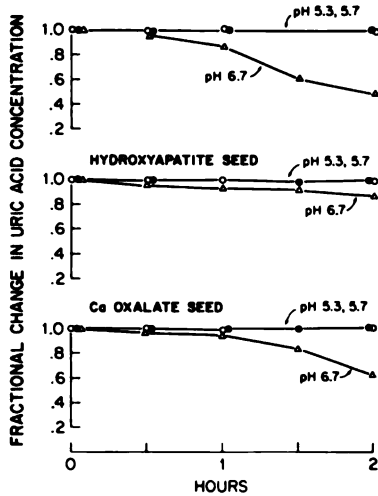


FIG. 1. Heterogeneous nucleation of urate by seeds of brushite, hydroxyapatite, and Ca oxalate. The seeds were added at time zero.

tration of uric acid did not change significantly in any of the test solutions. Seeding of solutions by uric acid (2 mg/ml) caused a prompt fall in filtrate concentrations of uric acid.

Uric acid is relatively stable at pH 5.3 and 5.7; however, it transforms to monosodium urate monohydrate at pH 6.7 in the presence of Na⁺ (3, 13, 17). Thus, if heterogeneous nucleation were to occur, the precipitates obtained at pH 5.3 and 5.7 will probably be uric acid, whereas that at pH 6.7 will be monosodium urate monohydrate. Seeding with brushite, hydroxyapatite, or Ca oxalate (indicated by time zero in the figure) did not cause crystallization of uric acid at pH 5.3

5.7, since the filtrate concentration of uric acid did not change. However, at pH 6.7, all three seeds caused a substantial fall in the filtrate concentration of uric acid. The analysis of precipitates disclosed presence in equimolar amounts of Na and uric acid. The results suggest that brushite, hydroxyapatite, and Ca oxalate induce heterogeneous nucleation of monosodium urate, but not of uric acid.

Heterogeneous nucleation of Ca phosphate (Fig. 2). Without seeding, there was no change in the filtrate concentrations of Ca and P in any of the solutions. When the solutions were seeded by brushite or hydroxyapatite (2 mg/ml), the filtrate con-

centrations of Ca and P decreased promptly. The results indicated that these solutions were metastably supersaturated with respect to Ca phosphate.

At pH 5.3, uric acid seeds were ineffective in inducing heterogeneous nucleation of Ca phosphate, since the filtrate concentration of Ca did not change. Since the pK_1 for uric acid has been reported to be 5.47 (17) and the principal cation in solution is Na⁺, uric acid seeds probably underwent partial transformation into monosodium urate at pH 5.7, 6.7, and 7.4 (13). At these pHs, the filtrate concentration of Ca declined after an initial lag period. The results indicate that monosodium urate, not uric acid, induces heterogeneous nucleation of Ca phosphate. This conclusion was supported by the demonstration that seeding by monosodium urate monohydrate causes a prompt fall in the filtrate concentration of Ca at all pHs. The analysis of the precipitates obtained at pH 5.3, 5.7, and 6.7 revealed presence in equimolar amounts of Ca and P, a finding which indicated the formation of dicalcium phosphate or brushite (7, 18). At pH 7.4, the Ca:P molar ratio of the precipitates was 1.4 ± 0.1 SD. The results suggested conversion of dicalcium phosphate toward apatite (7, 18).

The seed of Ca oxalate monohydrate was effective in inducing heterogeneous nuclea-

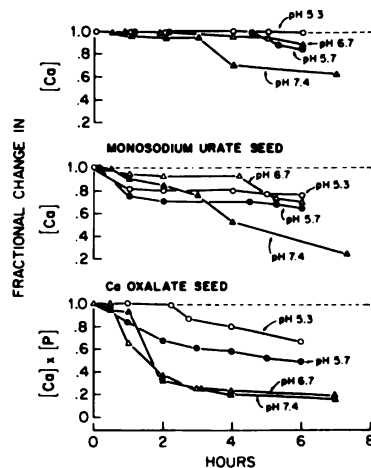


FIG. 2. Heterogeneous nucleation of Ca phosphate by seeds of uric acid, monosodium urate monohydrate, and Ca oxalate. The seeds were added at time zero. Brackets indicate molar concentrations.

tion of Ca phosphate at all pHs, since the concentration product of Ca and P of the filtrate decreased following seeding. However, Ca oxalate did not cause precipitation of Ca phosphate from solutions which were less supersaturated (approximately 2.5-fold saturated rather than 3.5-fold) with respect to brushite.

Heterogeneous nucleation of Ca oxalate (Fig. 3). Without seeding, filtrate concentrations of Ca and oxalate did not change in any of the solutions. The addition of Ca oxalate monohydrate (2 mg/ml) produced a rapid decline in the filtrate concentrations of Ca and oxalate.

Uric acid seed was incapable of inducing precipitation of Ca oxalate at pH 5.3 and 5.7, since the filtrate concentration of Ca did not change. However, at higher pHs in which the conversion of uric acid into monosodium urate may take place (17), seeding by uric acid caused a substantial decrease in the filtrate concentration of oxalate after a lag period of 3–4 hr. The seed of monosodium urate monohydrate was effective in lowering the filtrate concentrations of oxalate at all pHs. This decrease was less prominent at lower pHs, where monosodium urate is less stable (17). The Ca:Ox molar ratio or the precipitates closely approximated one. The results suggested that monosodium urate monohydrate induces heterogeneous nucleation of Ca oxalate, whereas uric acid does not.

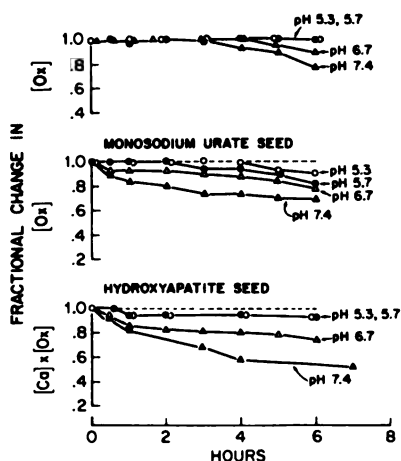


Fig. 3. Heterogeneous nucleation of Ca oxalate by seeds of uric acid, monosodium urate, and hydroxyapatite. The seeds were added at time zero.

Hydroxyapatite seed was effective at all pH's in causing heterogeneous nucleation of Ca oxalate, since the concentration product of Ca and oxalate of the filtrate declined following seeding.

Specificity of heterogeneous nucleation. Identical results were obtained when studies were carried out in siliconized glassware rather than in Pyrex glassware. Ground glass powder (5 mg/ml) did not provoke heterogeneous nucleation in any of the experiments. Although the solid:solution ratio of the precipitants was high, smaller amounts of precipitants often evoked heterogeneous nucleation, though usually to a lesser degree.

Heterogeneous nucleation of Ca oxalate by monosodium urate monohydrate and hydroxyapatite at pH 6.7 and 7.4 was also observed from solutions which were less supersaturated (3.5-fold rather than sixfold saturated) with respect to Ca oxalate.

Discussion. Heterogeneous nucleation requires demonstration that: (a) the solution under consideration is metastably supersaturated with respect to the precipitate (9), (b) the addition of homogeneous nuclei (identical in chemical composition to the precipitate) causes rapid precipitation, (c) seeding by heterogeneous nuclei (different in chemical composition from that of the precipitate) elicits precipitation, and (d) for heterogeneous nuclei which share a common ion with the precipitate, there is no significant dissolution of the nuclei and no consequent increase in the activity product with respect to the precipitate. In this study, these requirements were satisfied in the examination of heterogeneous nucleation which occurred with uric acid, monosodium urate monohydrate, calcium phosphates, and calcium oxalate.

One form of heterogeneous nucleation occurred between monosodium urate monohydrate and Ca salts. Thus, seeds of brushite, hydroxyapatite, and Ca oxalate monohydrate initiated precipitation of monosodium urate, but not uric acid. Conversely, the seed of monosodium urate monohydrate elicited nucleation of Ca phosphate and Ca oxalate, whereas the seed of uric acid did not.

Another form of heterogeneous nucleation, which involved Ca phosphates and Ca

oxalate, was identified. The seed of hydroxyapatite induced the nucleation of Ca oxalate. The seed of brushite was previously reported to possess similar action (9). Conversely, the seed of Ca oxalate monohydrate induced precipitation of Ca phosphate. The latter finding contradicts the report that Ca oxalate monohydrate is incapable of inducing heterogeneous nucleation of Ca phosphate (9). This discrepancy may be accounted for by the differences in the metastability of solutions utilized in the two studies. Thus, heterogeneous nucleation of Ca phosphate by Ca oxalate monohydrate could be demonstrated from solutions 3.5-fold saturated with respect to brushite, but not from those which were 2.5-fold saturated.

The various forms of heterogeneous nucleation described here could explain the formation of certain renal stones of mixed composition. By serving as *promoters* of nucleation, the heterogeneous nuclei may cause precipitation of mineral constituents from urine even though it may be metastably supersaturated with respect to the phases. The full significance of this study must await a better delineation of the physical chemistry of heterogeneous nucleation with the use of other complementary techniques, such as electron probe analysis (19), X-ray diffraction (1), and scanning electron microscopy (5).

Summary. Heterogeneous nucleation may constitute one of the mechanisms for the formation of renal stones of mixed composition. This process allows heterogeneous nuclei to initiate precipitation from solutions which are metastably supersaturated with respect to the precipitate. The seed of monosodium urate monohydrate, but not of uric acid, was capable of provoking nucleation of Ca phosphate and Ca oxalate. Conversely, seeds of brushite, hydroxyapatite, and Ca oxalate caused nucleation of monosodium urate, but not of uric acid. The seed of hydroxyapatite initiated precipitation of Ca oxalate, and vice versa. These physico-

chemical experiments provide a basis for the explanation of the formation of mixed stones.

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Effect of Estradiol on Uterine Weight, Thyroid Function, Food Intake, and Pituitary Weight of Genetically Obese (Fatty-Zucker) and Lean Rats (39486)

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The fatty rat inherits obesity as an autosomal Mendelian recessive trait (1). In previous publications (1-6), we have described small pituitaries (2), decreased thyroid function (3, 4), and impaired reproductive function in these animals (5). The abnormalities in reproductive function included: (1) a delayed vaginal opening; (2) a longer period between vaginal estrus cycles; and (3) small uteri (5, 6). The finding of small uteri suggested either that there were low levels of estrogen or that the uterus was less responsive to the circulating estrogen levels. The present experiments were designed to test the latter possibility. Three of the other abnormalities noted in the fatty rat might also result, at least in part, from a deficiency of estrogen. Food intake is reduced by treatment with estrogens and rises after bilateral oophorectomy (7). Second, thyroid function, as measured by the weight of the thyroid gland and the uptake of radioactive iodine, is increased by treatment with estrogens (8, 9). Low levels of estrogen might, therefore, decrease thyroid function. Finally, the weight of the pituitary is increased by high levels of estrogen and reduced by oophorectomy. The response of these systems as well as of uterine weight was used to test the response to estradiol.

Methods and materials. The experiments performed used genetically obese and lean female rats obtained from Dr. Lois Zucker (Stow, Massachusetts) or through breeding these animals in our laboratories. At the time of the experiment, the lean animals weighed 226 to 235 g and the fatty rats weighed 330 to 440 g, and they ranged in

age from 4 to 7 months. The rats were maintained singly in metabolism cages or in suspended wire bottom cages in groups of four rats. Laboratory chow was fed except for the last 3 weeks when a low iodine diet, purchased from Nutritional Biochemicals Company, was given (10).

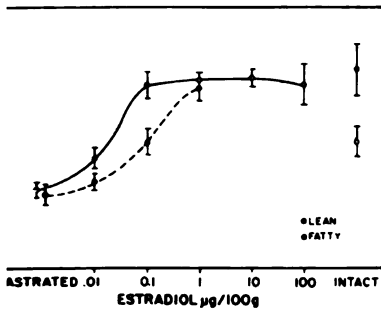
Twenty-six fatty and twenty lean rats were divided into groups of four rats each except for two groups of fatties which had only three rats (those receiving 0.01 $\mu\text{g}/100$ g or 100 $\mu\text{g}/100$ g of body wt). One subgroup of lean and obese rats was maintained as intact controls and the remainder were castrated. The protocol was similar to one published earlier (11). On the fifth day after castration, the various groups received injections of peanut oil or estradiol-17 β for a 3-day period, after which they were sacrificed. The estradiol-17 β was dissolved and diluted in peanut oil and given intramuscularly in doses ranging from 0.01 to 100 $\mu\text{g}/100$ g of body wt. Radioactive iodine uptake was determined by injecting 50 μCi of ¹²⁵I intraperitoneally 24 hr prior to sacrifice. Beginning 12 hr after the last dose of estradiol-17 β , pairs of rats, including one lean and one fatty, were killed until all animals had been sacrificed. The ovary (at castration or autopsy), the pituitary, and the thyroid were carefully removed and weighed, and the radioactivity in the thyroid was counted in a well-type scintillation counter. The Student's *t* test was used to compare group differences and significance was claimed if $P < 0.05$.

Results. In a preliminary experiment, groups of 10 fatty and 10 lean rats were castrated and half of the animals were treated with estradiol benzoate, 1 $\mu\text{g}/\text{day}$, for 1 week. The increase in uterine weight was smaller in the obese (fatty) rats than in the lean animals. To obviate the problem of

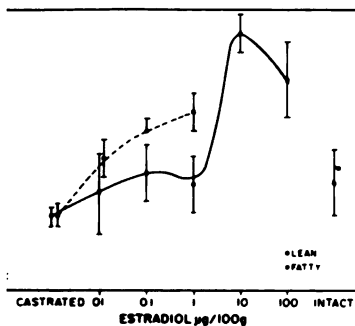
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lose of estradiol-17 β given to rats at different body weights, the present experiments designed using a log-dose treatment schedule in which estradiol-17 β was administered intramuscularly in doses ranging between 0.1 and 100 $\mu\text{g}/\text{body wt}$ (Figs. 1-3). There is a clear dose-response relationship between estradiol-17 β and the weight of the uterus (Fig. 1). Indeed, the genetically obese rats showed a somewhat greater uterine weight than did lean rats at low weight-related doses of estradiol. At 0.1 $\mu\text{g}/100\text{ g}$, the uterus of the fatty rats was significantly heavier than the uterus of the castrated lean animals with the same dose. With increasing estradiol-17 β , the weight of the uterus increased in both groups (Fig. 2).



Effect of estradiol on uterine weight of castrated rats. Rats were castrated 5 days before treatment and then treated for 3 days. Uterine weight (mean \pm SEM) against the dose of estradiol. Weights for intact rats are shown at the left end and the uterine weight of intact is shown at the right. The genetically obese rats appear to be slightly more sensitive to estradiol.



Effect of estradiol on the weight of the pituitary. The weight (mean \pm SEM) of the pituitary gland between lean and fatty rats. After castration, the pituitary was smaller, but increased in weight with treatment with estradiol.

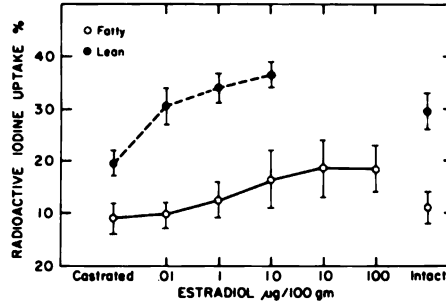


FIG. 3. Effect of estradiol on the uptake of radioactive iodine. The mean (\pm SEM) for 24-hr uptake of radioactive iodine is plotted against the dose of estradiol. The genetically obese rats had lower thyroidal uptake of radioiodine throughout, but estradiol enhanced the uptake in both obese and lean animals.

Estradiol-17 β also increased the uptake of radioactive iodine by the thyroid in lean and fatty rats (Fig. 3). At each dose of estradiol-17 β , however, the lean rats had a significantly higher uptake of radioactive ^{125}I than the fatty rats. The mean weight of the adrenal glands was heavier in the obese animals (6), but this difference was not significant and there were no significant effects on the weight of this gland by treatment with estradiol-17 β . Food intake in the control periods and after castration usually ranged from 13 to 15 g/day in the lean animals. After treatment with the highest dose of estradiol-17 β , there was a significant drop in food intake in the lean animals but no effect from the lower doses. The fatty rats ate more food (16-20 g/day) than the lean animals, and even the highest dose of estradiol-17 β did not decrease food intake. The highest dose of estradiol-17 β significantly reduced the body weight of the fatty rat by 30%, but the lower doses did not.

Discussion. The present experiments confirm that the uteri of the fatty rats are significantly smaller than the uteri from lean animals (2, 5, 6). Similar atrophic changes in the uterus have also been observed in most other genetically obese rodents (1). The rise in uterine weight in response to exogenous estradiol-17 β is normal in the fatty rat and the other obese rodents (1). This suggests that the reduced uterine weight in the fatty rat may result from a low concentration of circulating estradiol-17 β . Attempts to measure this hormone by the radioimmuno-

noassay system for estradiol-17 β available in our laboratory were unsuccessful in distinguishing between normal and low circulating levels of this estrogen. However, indirect evidence supports the contention that the fatty rat is hypoestrogenized. First of all, we found that over the dose range from 0.01 to 100 μ g of estradiol-17 β /100 g of body wt, the increase in uterine weight of the fatty rats occurred at the same time or at a slightly lower dose of estradiol than that found in the lean controls. We could thus find no indication of a reduced sensitivity of the uterus of fatty rats to exogenous estrogens. However, the uterine weight of the intact fatty rats was significantly lower than that of the lean animals and was comparable to the weight of the uterus of rats treated with a daily dose of between 0.01 and 0.1 μ g of estradiol-17 β . This suggests that ovarian production of estrogenic hormones in the fatty rat may be lower than in the lean rat.

Changes in the weight of the pituitary and in thyroidal uptake of radioactive iodine were also observed during treatment with estradiol. The pituitary of the fatty rat is usually smaller than in lean animals (1, 4-6). This was also observed in the preliminary study noted above, but was not demonstrated in the large dose-response study (see Fig. 2). Had larger numbers of rats been used, however, we would have anticipated a difference in pituitary weight between intact fatty and lean rats. After castration, the weight of the pituitary in the lean and fatty rats was identical. During treatment with estradiol, the weight of the pituitary increased in both groups, but at different doses. It is thus possible that the small pituitary usually observed in the fatty rat may result in part from low circulating concentrations of estradiol. It is also possible that some of the differences in uptake of radioactive iodine between lean and fatty rats (4, 6) may also result from differences in the levels of circulating estradiol-17 β . The rise in thyroidal uptake of radioactive iodine after treatment with estradiol-17 β is similar to data reported in the literature (8-10). Regulation of food intake in the fatty rat differs in several respects from its control in lean animals (11). Fatty rats adapt to changes in

the caloric density of their diet but rats with obesity and hypothalamic injury do not. In the cold, the food intake of fatty rats falls, but the lean rats compensate for heat loss by eating more (12). The present experiments show a third difference in food intake between fatty and lean rats. Treatment of lean rats with estradiol-17 β , 1 μ g/day, reduced food intake. This dose, however, did not alter the food intake of the fatty rats.

Summary. Genetically obese (fatty) and lean rats were treated with several doses of estradiol-17 β . The uterus of the untreated fatty rats was significantly smaller than the uterus of the lean rats. The uterine weight of castrated lean or fatty rats increased in weight after treatment with 0.1 or 1.0 μ g/day of estradiol-17 β , but doses of 10 or 100 μ g/day produced no further effect. Although castration lowered the uptake of radioactive iodine by the thyroid of lean rats, it was still significantly higher than in the thyroid of fatty rats. Thyroidal uptake of radioiodine also increased with estrogen treatment in both groups but the lean animals were more sensitive. We concluded that the small uterus in the fatty rat may be due to decreased estrogenization and that this may also partly account for the small pituitary and low thyroidal uptake of radioiodine.

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Plasma Volumes and Entrapment of Plasma in Tissues of Normal and Insulin-Immunized Guinea Pigs¹ (39487)

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After intravascular injection, a substance will disappear from the blood to accumulate or to be metabolized within one or more tissues or to be excreted from the body. If found within a given tissue after death, that substance could be situated within the cells or in the extracellular spaces of that tissue. When assessing the fate of insulin injected into guinea pigs it therefore became important to know the plasma volumes of the injected animals and the amounts of plasma trapped within the livers, kidneys, and spleens in which the hormone was found after death. The following is an account of a study in which 115 guinea pigs were injected with mixtures of radio-iodinated albumin and insulin. The fate of the albumin was followed for periods of up to 2 hr. The results show that previous estimates of plasma volume in the normal guinea pig were probably low and that significant amounts of plasma do remain trapped in the livers, kidneys, and spleen after death.

Materials and methods. Guinea pigs. Male albino guinea pigs (493-1445 g) were fed a standard diet *ad libitum* and had free access to water at all times. They included 67 animals which had received no previous treatment (normal) and 48 (immune) which had been immunized by a standard method (1) with bovine or porcine insulin.

Inocula. Each animal received an inoculum which contained a commercial preparation of ¹³¹I-labeled human serum albumin (Albutope, E. R. Squibb and Sons, Inc., N.J.) with a specific activity of 20 to 40 μ Ci/mg of protein. This was added in a final concentration of about 10 μ Ci/ml to a buffered solution (3% NGPS; see below) which also contained commercially labeled

¹²⁵I-labeled porcine insulin (approx 10 μ Ci/ml) and normal or anti-insulin guinea pig serum (0.2-0.7 ml of serum/ml of inoculum).

Experimental procedure. Each animal was anesthetized with pentobarbital (25-50 mg/kg) and given a single subcutaneous injection of antihistamine (chlorpheniramine maleate injection, N.F.; 10 mg). Catheters (PE 90, id, 0.034 in.; Clay Adams, N.J.) were inserted in the external jugular vein and carotid artery, both being connected by way of three-way taps to a pump used to infuse heparinized saline (10 U/ml, sodium heparin injection, U.S.P.) at a steady rate (0.1 ml/min). After about 15 min, the inoculum (1.0 ml) was injected through the venous catheter, the volume being carefully measured and residual inoculum in the catheter being flushed into the animal with saline immediately after injection. From the arterial catheter a total of three to 11 measured samples of blood (0.8 or 1.0 ml) was drawn at timed intervals from 1 min to 2 hr after each injection. Care was taken to ensure that blood samples were not contaminated with saline from the catheters or taps and that plasma was rapidly separated by centrifugation. At the end of each experiment, blood was drawn from the arterial catheter (more than 25 ml) until all respirations ceased; a lethal dose of barbiturate was given if signs of life persisted. The abdomen was then opened and the liver, kidneys, and spleen were removed and weighed.

Assay of radioactive contents of inocula and tissues. Weighed portions of liver, kidney, and spleen (ca. 1.0 g) were placed in ice-cold distilled water (10 ml) and homogenized (Polytron, Kinematica GMBH, Lucerne, Switzerland). Aliquots of inocula and plasma were diluted in a neutral phosphate buffer (0.05 M, Na₂HPO₄/KH₂PO₄; pH 7.0) containing sodium chloride (0.4%, w/

¹ This study was supported by U.S. Public Health Service Grant AM-16534.

² Author to whom reprint requests should be addressed.

1 normal guinea pig serum (3%, v/v); solution is here termed 3% NGPS. A (0.3 ml) was diluted in 3% NGPS (10 ml). Using a micropipet, (Eppendorf, Lab. Laboratories, N.Y.) a small aliquot of inoculum (10 μ l) was diluted in 3% NGPS (5.0 ml). Aliquots (0.5 ml) of these diluted samples of plasma and inoculum and well-mixed homogenates were placed in all culture tubes (10 \times 75 mm) and (3/4 or 6/8, 75%) of the replicate tubes were treated with an equal volume of chloroacetic acid (0.5 ml; 20 TCA, w/v). Resultant precipitates, after centrifugation and aspiration of supernatant fluid, were washed once with more dilute acid (10 ml; 10% TCA, w/v). The radioactive contents of these washed precipitates (TCA-precipitable radioactivity) and of the undiluted samples of inocula, plasma, and homogenates (total radioactivity) were measured in an automatic gamma counter (Model 1195, Searle Analytic Inc., Des Plaines, Ill.). All values for radioactivity (counts) quoted below refer to ^{131}I in TCA-precipitates from inocula, plasma, and homogenates and accounted, in each case, for more than 95 to 97% of the total radioactivity contents of these tissues. ^{125}I in the insulin did not interfere with these measurements.

Calculations. Plasma volumes (PV) were calculated from the amount of injected precipitable radioactivity (C_i) and the initial concentration of radioactivity in plasma at zero time (C_p^0/v):

$$\text{PV} = C_i \times v / C_p^0, \quad (1)$$

where v is the volume of plasma (0.0536 ml) used for radioactive content at each time interval. The value of C_p^0 was determined by extrapolation to zero time of the line joining the radioactive contents of plasma samples (C_p^t) to the times (t , min) at which they were collected:

$$\log_{10} C_p^t = \log_{10} C_p^0 - bt, \quad (2)$$

where b is a regression coefficient.

The volume of distribution of injected albumin (AV) was calculated on the assumption that the only losses of albumin from the body were those due to successive bleedings. The amount lost at each such

bleeding (C_{bl}) is given by the expression:

$$C_{bl} = (100 - \text{Hct})C_p^t \times V_{bl}/100v, \quad (3)$$

where V_{bl} is the volume of blood drawn (0.8 or 1.0 ml) and Hct is the hematocrit. At any time t after injection of the inoculum, the volume of distribution of the residual labeled albumin (AV'), the albumin space, is

$$\text{AV}' = (C_i - \sum C_{bl}) \times v / C_p^t \quad (4)$$

The hematocrit (Hct) was not measured at any time but was assumed to be 40%, a value somewhat less than the range usually quoted (41 to 48%) for normal guinea pigs (2-4) but reasonable for animals that were bled repeatedly over periods of up to 2 hr. For comparative purposes, the albumin spaces of individual animals are expressed as percentages of their plasma volumes ($100 \times \text{AV}'/\text{PV}$).

The plasma contents of individual tissues examined after death (PV_T) are given by the expression:

$$\begin{aligned} \text{PV}_T &= \text{Total radioactive content of tissue} - \text{radioactive content of plasma} \\ &= [C_H \times W(10 + w)/0.5 \times w] / [C_p^d/v], \quad (5) \end{aligned}$$

where w is the weight of the portion of tissue homogenized in water (10 ml), W is the weight of the whole tissue, and C_H and C_p^d are the radioactive contents (respectively) of the aliquot (0.5 ml) of tissue homogenate and of the sample of plasma (volume, v) taken at the time of death. It was assumed, therefore, that all radioactive albumin found in a tissue is confined to the plasma.

Correlations and comparisons based on such data were carried out by methods described by Snedecor and Cochran (5).

Results. Plasma volumes. Of 112 determinations, 15 have been arbitrarily excluded from present consideration because in these instances the standard errors of estimates of $\log_{10} C_p^0$ [see Eq. (2)] exceeded 1.5% of the estimates themselves. Of the remainder, 60 were for normal and 37 for insulin-immunized guinea pigs.

The plasma volumes of normal and insulin-immunized guinea pigs increased with

total body weight but when related to unit body weight (ml/100 g) they decreased (Table I). Linear relationships were established in both groups of animals between plasma volume and body weight (Table II, lines 1 and 2) but in neither case did the line pass through the origin; this accounts for the decrease in the ratio of plasma volume to body weight as body weight increases (Table I). The slopes (b) of these two regression lines do not differ significantly ($P > 0.05$) but, as illustrated in Fig. 1, the elevation of the line for immune animals is significantly higher ($P < 0.01$) than that for normal guinea pigs.

Linear relationships were established for 18 normal and 18 insulin-immunized guinea pigs between albumin space and time for all samples of blood drawn more than 10 min after inoculum injection (Table II, lines 3 and 4); they do not differ significantly in any respect ($P > 0.05$). When the results for the two groups of animals are combined (Fig. 2), the albumin space is seen to increase

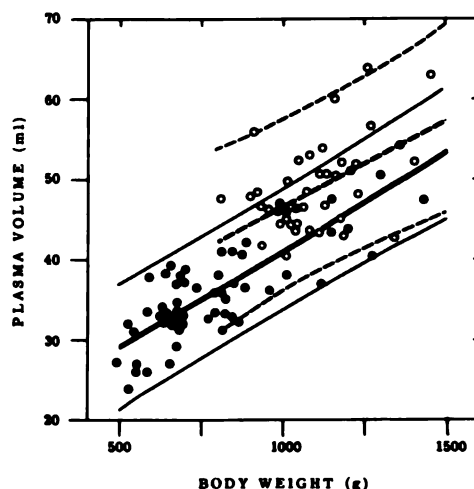


FIG. 1. Plasma volumes (ml) and body weights (g) of normal (closed circles) and insulin-immunized (open circles) guinea pigs. Also shown are the corresponding regression lines (heavy print) and limits of confidence (95%) for individual observations (light print) for normal (continuous lines) and immune (interrupted lines) animals.

TABLE I. PLASMA VOLUMES AND BODY WEIGHTS OF NORMAL AND INSULIN-IMMUNIZED GUINEA PIGS

GP ^a group	Normal ^b				Immune ^b			
	n	Body wt (g)	Plasma volume		n	Body wt (g)	Plasma volume	
			ml	ml/100 g			ml	ml/100 g
A	28	627 ± 12	32.5 ± 0.8	5.18 ± 0.10				
B	17	814 ± 10	36.1 ± 0.8	4.43 ± 0.09	2	854 ± 46	42.7 ± 5.2	4.97 ± 0.33
C	6	997 ± 11	43.3 ± 2.0	4.33 ± 0.18	18	1006 ± 13	46.6 ± 1.0	4.65 ± 0.12
D	7	1200 ± 25	44.7 ± 2.0	3.73 ± 0.15	14	1174 ± 14	51.2 ± 1.6	4.36 ± 0.12
E	2	1395 ± 35	50.8 ± 3.4	3.65 ± 0.33	3	1395 ± 32	52.6 ± 5.8	3.76 ± 0.33

^a Animals are grouped (n) according to ranges of body weight in grams (A, 500–700; B, 701–900; C, 901–1100; D, 1101–1300; and E, over 1300).

^b All quoted values refer to means (\pm SE).

TABLE II. LINEAR INTERRELATIONSHIPS FOR GUINEA PIGS INJECTED WITH ¹³¹I-LABELED HUMAN SERUM ALBUMIN^a

Line	Animal group	Ordinate (y)	Abscissa (x)	Observations (n)	Intercept (c)	Regression coefficient ($b \pm S_b$)	Correlation coefficient (r)
1	Normal	Plasma volume	Body weight	60	17.11	24.0984 ± 2.1468	0.8275
2	Immune	(ml)	(kg)	37	24.88	21.7165 ± 6.1127	0.5148
3	Normal	Albumin space	Time (min)	149	1.9951	0.00114 ± 0.00009	0.7201
4	Immune	(Log ₁₀ % of PV)		162	1.9955	0.00104 ± 0.00008	0.7358
5	All ^a	Liver weight	Body weight	87	6.96	22.3768 ± 1.3078	0.8803
6		Kidney weight	(kg)	87	0.80	4.4297 ± 0.3628	0.7980
7		Spleen weight (g)		82	0.50	0.6598 ± 0.1470	0.4485
8	All ^a	Liver plasma	Tissue weight	87	0.28	0.0972 ± 0.0057	0.8804
9		Kidney plasma	(g)	87	-0.25	0.2026 ± 0.0098	0.9135
10		Spleen plasma (ml)		80	0.00	0.0951 ± 0.0084	0.7897

^a The symbols refer to those for a linear regression ($y = C + bx$), the number of paired observations used for calculation (n), the standard errors of the regression coefficients (S_b), and the correlation coefficients (r). Where Regression lines for normal and immune animals were not significantly different ($P > 0.05$), they have been combined (*). Other statistical comparisons are considered in the text.

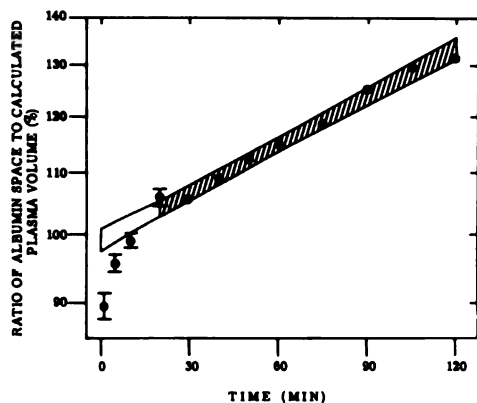


FIG. 2. Ratios of albumin space to plasma volume ($100 \times AV/PV$) at timed intervals after injection of radio-iodinated albumin. Each point represents the mean of 25 to 36 observations for 18 normal and 18 insulin-immunized guinea pigs. Also shown are the standard errors for early mean values (1 to 20 min) and a shaded area covering the limits of confidence (95%) for the combined observations after 10 min for normal and immune animals (see Table II, lines 3 and 4).

after 10 min at a rate of about 17%/hr. Mean values (\pm SEM) for albumin space (relative to a plasma space of 100) at 1 (90.3 ± 1.3), 5 (95.8 ± 1.0), and 20 (106.1 ± 1.3) or more minutes after injection are all significantly different from the calculated plasma volume ($P < 0.001$). Only at 10 min is the albumin space (99.0 ± 1.0) equal to the plasma volume ($P > 0.05$).

Plasma entrapment by tissues. Linear relationships were established for 87 guinea pigs between the weights of the livers, kidneys, and spleens and the body weights of the animals from which they were obtained, and between the plasma contents and weights of the organs themselves. Since no distinction could be made between the various regression lines for normal and insulin-immunized animals, the results have been combined (Table II, lines 5 to 10). One or other of the organs from the remaining 25 guinea pigs, 12 of which were normal and 13 of which were immunized, were arbitrarily considered to have abnormally high plasma contents; contents (milliliters of plasma per gram of tissue) exceeded the mean values for animals of comparable weight by more than two standard deviations. As illustrated in Fig. 3, the livers, kidneys, and spleens of these 25 guinea pigs, with minor exceptions,

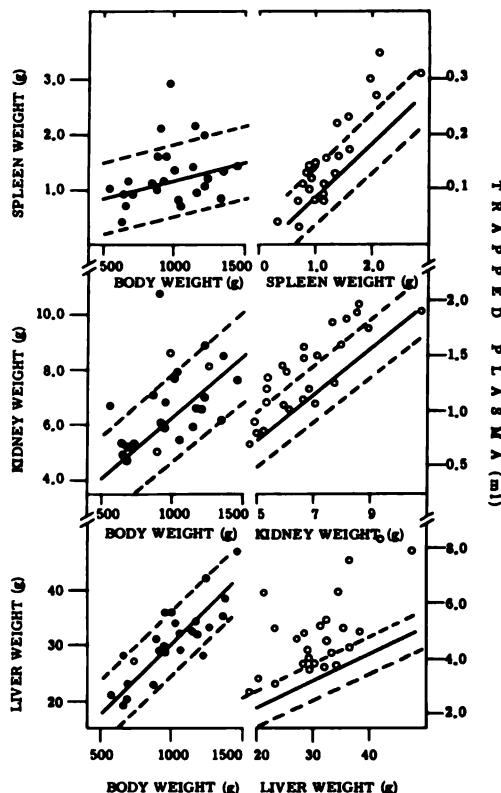


FIG. 3. The organ weights (g, closed circles) and the volumes of trapped plasma (ml, open circles) for the livers, kidneys, and spleens of 25 guinea pigs considered to have high plasma tissue contents. The regression lines (continuous) and limits of confidence (95%) for individual observations (dotted lines) refer to values obtained from 87 other animals. In each case, tissue weight or trapped plasma have been related to body weight (g) or tissue weight (g). The values shown here are derived from information shown in Table II.

were not excessively heavy relative to body weight. However, the vast majority had tissues whose plasma contents exceeded the mean values for livers, kidneys, and spleens from the other 87 guinea pigs. Contents were abnormally high in 17 livers, 11 pairs of kidneys, and 8 spleens. In eight animals the liver was the only affected organ, but in nine other animals the kidneys ($n = 3$), spleen ($n = 5$), or both of these organs ($n = 1$) were also affected. In five animals the kidneys only were affected, but in two others the kidneys and the spleen were involved. The spleen was never the only organ affected. Of the 17 animals whose livers were affected, eight had died (usually from

respiratory complications) before they could be exsanguinated; no note of such complications was made in the cases of the other nine animals with affected livers.

Discussion. Investigation of the vascular spaces in guinea pigs began in 1929 when Went and Drinker (6) first used Vital Red to estimate blood volume. Since then, others (2, 3, 7, 8) have used dyes, radioiodinated proteins, and radiolabeled red cells to estimate blood volume but have reported only indirect estimates of plasma volume. Constable (9) bled guinea pigs 4 min after injection of Evans Blue (T1824) and reported that plasma volume falls from a value of 5.73 ml/100 g body wt at birth to 3.0 ml/100 g body wt in guinea pigs weighing 800 to 900 g. Edmondson and Wyburn (10) bled their guinea pigs 5 min after injection of radioiodinated albumin. For animals weighing about a kilogram, they found that mean plasma volumes (\pm SEM) for males (3.31 ± 0.07 ml/100 g body wt) and females (3.59 ± 0.07 ml/100 g body wt) were about the same. Using the same technique, Wiseman and Irving (4) got a slightly larger value (4.1 ± 0.2 ml/100 g body wt) for animals weighing 600 g. Rieke, in an unpublished observation reported by Osmond and Everett (3), without experimental details, obtained a similar plasma volume (4.1 ml/100 g body wt) for even smaller animals weighing about 300 g. By comparison, as shown in Table I, the plasma volumes of the present animals were greater and decreased from about 5.2 ml/100 g body wt for those weighing about 600 g to a significantly lower value of 3.7 ml/100 g of body wt for guinea pigs weighing about 1200 g. The discrepancy between these and previously published findings is probably due, at least in part, to differences in technique. Thus, the present results are based on multiple observations made between at least 10 and as much as 120 min after injection of labeled albumin, a method which is "to be preferred and recommended" (11) for the estimation of plasma volume in man. Plasma volumes determined by this method, as illustrated in Fig. 2, are likely to exceed any which depend upon single observations on blood samples drawn less than 10 min after injection, the time of sampling used by all the investigators

quoted above. It should also be noted that the present relatively larger volumes are of the same order as those (4 to 6 ml/100 g body wt) reported for other small mammals (12).

No explanation can yet be given for the slightly but significantly larger plasma volumes of insulin-immunized guinea pigs. The difference does not appear to be due to increased permeability of capillary membranes induced by insulin, which was present in all inocula. Had this been so, the labeled albumin should have escaped from the plasma more rapidly and so increased the albumin space more rapidly in immune than in normal guinea pigs. This did not occur, as illustrated in Table II (lines 3 and 4), and was probably prevented by the administration of antihistamine. It was also considered possible that the immune animals may have been more lean (less obese) than normal animals of comparable total body weight and that this could have led to a relative increase in their plasma volumes. This, however, also seems unlikely because the livers, kidneys, and spleens of the immune animals were no larger than those of the normal guinea pigs of comparable weight (Table II, lines 5 to 7).

Among the 87 guinea pigs which had been adequately exsanguinated at the time of death in the present studies, significant volumes (mean \pm SEM, milliliters per gram of tissue) of plasma remained in the livers (0.108 ± 0.001), kidneys (0.158 ± 0.002), and spleens (0.090 ± 0.002). A similar distribution was reported in dogs by Gibson *et al.* (13), who also found higher concentrations in the kidneys (0.17 ml/g) than in the livers (0.12 ml/g) or spleens (0.07 ml/g) under similar circumstances. These levels probably do not reflect the entrapment of plasma alone because it is known that albumin escapes from the plasma into the lymph, especially in and around the liver (14); such leakage also accounts for the increase in the albumin space seen over 2 hr in the present studies (Fig. 2). Significantly higher levels of entrapment were found in the livers (0.160 ± 0.008), kidneys (0.198 ± 0.006), and spleens (0.131 ± 0.018) of the other 25 guinea pigs used here. The liver was the organ most affected (17/25) and in

such cases (8/17) it was noted that the animal had died before it could be exsanguinated. Such increased entrapment of plasma was not reflected in the weights of affected organs and was only detected by direct measurement (Fig. 3). The present

therefore shows that significant amounts of blood can become trapped at the time of death in the livers, kidneys, and spleens of guinea pigs and that such entrapment can become excessive if the animal is exsanguinated at the time of death. These facts should be taken into account when studying the fate of any substance, endogenous or exogenous, which is present in the blood in high concentrations and appears to become concentrated in a particular organ, such as the liver, where blood could pool after death.

Summary. Relative to body weight, the plasma volumes of 60 normal guinea pigs ranged from 5.2 to 3.7 ml/100 g body wt as body weight increased from about 600 to 1000 g. Lower values reported by others are attributed to differences in methodology. Similarly, higher plasma volumes were found in insulin-immunized guinea pigs. After exsanguination at the time of death, trapped plasma accounted for 10 to 20% of the plasma in the livers, kidneys, and spleens, plasma being trapped when death occurred and before exsanguination could be effected.

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Induction of Erythroid Colony Forming Cells (CFU-E) in Murine Spleen by Endotoxin¹

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The plasma clot culture system containing erythropoietin (Ep) permits erythroid precursors from bone marrow or spleen to form *in vitro* small colonies of hemoglobin-synthesizing erythroblasts (1). Early erythroid precursors (committed erythroid stem cells) most likely comprise a multistage compartment that is interposed between the pluripotential stem cells (CFU) and the morphologically recognizable proerythroblasts (2, 3). The position of the colony-forming erythroid precursors (CFU-E) within this compartment and the factors regulating or influencing their population size are not exactly known. Erythropoietin injection into hypertransfused mice has been shown by Gregory *et al.* (4) to increase the number of femoral or splenic CFU-E, but increasing the concentration of Ep in the medium above a plateau level failed to increase colony formation. The injection of endotoxin into mice is known to increase their splenic erythropoiesis as measured by ⁵⁹Fe incorporation (5, 6), and the present study was undertaken to examine the effect of endotoxin on the number of splenic CFU-E in the presence or absence of Ep. It will be shown that endotoxin injection indeed induced splenic CFU-E in normal mice and in mice whose endogenous erythropoietin production was suppressed by posthypoxic polycythemia. Differences in increases in CFU-E between those induced by Ep and those induced by endotoxin will be discussed.

Methods. Female CF₁ mice of from 23 to 27 g body weight were used in groups of eight. Mice were made polycythemic by 3 weeks of exposure (18 hr/day) to an atmospheric pressure of from 380 to 340 mm Hg. Endotoxin (lipopolysaccharide B, *S. typhosa*, Difco) was diluted in normal saline and injected ip. Human erythropoietin (56

units/mg) was obtained through the Erythropoietin Committee of the NIH Lung Institute. The Ep was processed by the Hematology Research Laboratories of the University of California at Los Angeles. The Ep was dissolved in 0.94 g% sodium chloride solution and injected iv in doses as indicated.

For CFU-E measurement, spleen was removed at intervals as indicated, and the last injection of either Ep or endotoxin. The spleen was cut in small pieces and a suspension was prepared in supplemented Eagle's MEM (Grand Island Biological Company) by drawing the cells back through a 19-gauge needle. An aliquot was used for counting in a Coulter Counter using a 10% (v/v) solution of erythrocytes. The number of spleen CFU-E was measured by modification of the 2-day plasma clot culture method of McLeod *et al.* (1). The culture medium consisted of 2 ml of α-MEM (Flow Laboratory, Rockville, Md.), 0.1 ml of a 10⁻³ M solution of thioglycerol (9), 1.5 ml of heat-inactivated (30 min, 60°) fetal calf serum (Gibco), 0.25 ml of detoxified (1) bovine serum albumin solution (20%), 0.055 ml of L-glutamine solution (2 mg/ml), 0.17 ml of embryo extract (GIBCO), and 0.5 ml of Ep (dissolved in α-medium) per ml of medium. A measured aliquot of each cell suspension was added to a total of 1.5 ml of medium and was plated in 6-well microwells (0.1 ml/well). The cell concentration ranged from eight to ten 10⁵ per ml of medium. After incubation for 48 hr in air plus 5% CO₂, the clots were transferred to slides, stained with benzidine hematoxylin, and colonies of eight or more benzidine positive cells were scored.

In some experiments a portion of the suspension (3 × 10⁶ cells) was transferred to slides by means of cytopsin centrifuge preparations were stained with Giemsa in the presence of proerythroblasts was

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o + 4 by two observers.

splenic ^{59}Fe incorporation into heme measured by iv injection of $1.5 \mu\text{Ci}$ of ^{59}Fe 1 hr after Ep or the last endotoxin injection. Six hours later a cell suspension of spleen was made as described above. The cell suspension was washed in saline and the cells were ruptured through re-freezing and thawing. One milliliter of 1% formalin's solution was then added and the suspension was allowed to stand overnight. The stromata were removed by high speed centrifugation. The supernatant was then adjusted to 2.0 and the heme was extracted by shaking with methyl ethyl ketone. Results are expressed as the percentage of the injected ^{59}Fe present in the spleen heme. The Student's *t* distribution was used to assess statistical significance.

Results. The number of CFU-E per spleen was 82 to $110 \cdot 10^3$ in normal mice and 21 to $46 \cdot 10^3$ in polycythemic mice on the fifth posthypoxic day. Table I shows the effects of a single injection of 5 to $10 \mu\text{g}$ of endotoxin on splenic CFU-E 2 hr later and on the 6-hr splenic incorporation into heme measured 96 hr after endotoxin injection. Ten micrograms or more induced significant ($P < 0.05$) increases in CFU-E, whereas the percentage increase in the *in vivo* ^{59}Fe incorporation was much smaller and was only significant after injection of $50 \mu\text{g}$ of endotoxin. Erythroid colonies developed when spleen cells from normal or from endotoxin-injected mice were cultured without Ep in semisolid medium.

Table II shows the progressive increase in CFU-E in normal and in polycythemic (6 days posthypoxia) mice after 2, 4, and 6 days of injection of $10 \mu\text{g}$ of endotoxin per day. In the normal mice a 40-fold increase in CFU-E was found after six injections and a 21-fold increase was found in polycythemic mice. The absolute number of CFU-E in the endotoxin-treated polycythemic mice was more than eight times

TABLE II. EFFECT OF DAILY INJECTIONS OF $10 \mu\text{g}$ OF ENDOTOXIN ON CFU-E AND 6-HR ^{59}Fe HEME INCORPORATION IN SPLEENS OF NORMAL AND POLYCYTHEMIC MICE^a

Days of endotoxin	Normals		Polycythemic	
	CFU-E $\cdot 10^{-3}$	^{59}Fe (%)	CFU-E $\cdot 10^{-3}$	^{59}Fe (%)
0	85	1.04	34	0.013
2	459	2.23	186	0.019*
4	1554	3.44	472	0.014*
6	3997	4.53	715	0.021*

^a Eight mice per group.

* Not significant ($P > 0.05$).

greater than that in untreated normal, i.e., nonpolycythemic, mice. The splenic ^{59}Fe incorporation was markedly different in the two groups. The progressive rise in splenic iron incorporation seen with endotoxin treatment in the nonpolycythemic mice was absent in the mice whose endogenous production of erythropoietin had been suppressed by polycythemia. Injection of 1 unit of erythropoietin 1 day after termination of the six endotoxin injections induced an increase in splenic ^{59}Fe incorporation from 0.027 to 0.86%, indicating that the endotoxin-induced precursors were capable of *in vivo* development into heme-synthesizing erythroblasts. In the absence of erythropoietin, however, the endotoxin-induced precursors were not transformed *in vivo* into morphologically identifiable proerythroblasts. In the experiment presented in Table III, cytopsin preparations of spleen cell suspensions were examined for the presence of proerythroblasts at various times after injection of Ep or of endotoxin into posthypoxic polycythemic mice. Very few proerythroblasts were seen in the spleens of polycythemic mice on the fifth posthypoxic day, and no significant increases occurred after endotoxin injection. Ep, in contrast, induced within 16 to 24 hr a sizable cohort of splenic proerythroblasts.

Table III also shows a significant differ-

I. EFFECT OF ENDOTOXIN DOSE ON CFU-E AND 6-HR ^{59}Fe HEME INCORPORATION IN SPLEENS OF NORMAL MICE^a

	Endotoxin dose (μg)				
	0	5	10	30	50
CFU-E $\cdot 10^{-3}$	102 ± 21	188 ± 38	272 ± 38	397 ± 97	467 ± 121
^{59}Fe (%)	1.04 ± 0.23	0.96 ± 0.06	1.07 ± 0.31	1.58 ± 0.25	2.17 ± 0.41

mice per group; mean \pm SEM.

TABLE III. EFFECT OF A SINGLE INJECTION OF 30 μ g OF ENDOTOXIN OR OF 1.5 UNITS OF ERYTHROPOIETIN ON CFU-E AND PROERYTHROBLASTS (PE) IN SPLEENS OF POLYCYTHEMIC MICE^a

Time of injection before culture (hr)	Erythropoietin		Endotoxin	
	CFU-E ^b · 10 ⁻³	PE	CFU-E ^b · 10 ⁻³	PE
0	38 ± 13	±	38 ± 13	±
16	156 ± 50	++	51 ± 10	±
24	154 ± 23	++++	87 ± 25	±
48	102 ± 22	+++	162 ± 25	±
72	32 ± 12	±	201 ± 21	±
96	30 ± 16	±	249 ± 36	±
120	34 ± 17	±	79 ± 21	±

^a Eight mice per group.

^b Mean ± SEM.

ence between Ep and endotoxin in the time course of CFU-E induction. Ep injection resulted in a maximal increase at 16 hr after its injection, and the CFU-E returned to base line levels at 72 hr after Ep. In contrast, no significant splenic CFU-E increases were found at 16 or 24 hr after endotoxin, and maximal increases occurred between 72 and 96 hr after its injection.

Discussion. The presented data show that endotoxin induced in the spleen of normal or of polycythemic mice erythroid precursor cells which differentiated *in vitro* in the presence of Ep into colonies of hemoglobin-containing erythroblasts. When left in the intact mouse whose endogenous Ep was suppressed by polycythemia, these precursors did not differentiate into identifiable proerythroblasts or later stages. These findings present clear confirmation of the capability of the plasma clot culture system to induce *in vitro* differentiation of erythroid precursors. In the nonpolycythemic mouse, the endotoxin-induced precursors differentiated *in vivo*, and this resulted in increases in splenic ⁵⁹Fe incorporation into heme. Injection of Ep into endotoxin-treated polycythemic mice achieved the same effect. The induction of splenic CFU-E by endotoxin thus did not require the presence of Ep, but the transformation of CFU-E into erythroblasts required its presence both *in vivo* and *in vitro*.

The number of splenic CFU-E in polycythemic mice could also be markedly increased by an injection of Ep, and our results thus confirm earlier findings of Gregory (4).

The different time element in the induction of CFU-E by endotoxin versus that by Ep suggests different modes of action. Maximal CFU-E increases occurred from 72 to 96 hr after injection of endotoxin. Endotoxin has been shown to turn noncycling CFU into cell cycle (7) and to increase the number of splenic CFU (8). It would seem plausible, therefore, that the relative late increase in CFU-E was the result of a proliferation-stimulating effect of endotoxin on either the pluripotential or the committed erythroid stem cells. Erythropoietin, in contrast, induced maximal CFU-E numbers within 16 hr of its injection. A number of observations (5, 6) indicates that Ep not only causes *in vivo* transformation of precursors (ERC) into proerythroblasts, but that it enhances at the same time the development of earlier precursors into ERC. The egress of ERC through their erythropoietin-regulated transformation into proerythroblasts is thus balanced, and the population size of ERC is maintained at various rates of erythropoiesis. It is reasonable to assume that the CFU-E are identical or closely related to the ERC, which are regarded to represent *in vivo* the immediate precursors of proerythroblasts. A single injection of Ep in the polycythemic mice increased the splenic CFU-E within 16 hr by 400%. This increase probably is the result of a recruitment of earlier precursors and it thus corresponds to the recruitment of immediate proerythroblast precursors (ERC) observed in the intact mouse. However, in view of the rather large increase occurring within a time span of 16 hr, an alternative explanation can be entertained, namely, erythroid colony formation by proerythroblasts in addition to the colonies formed by their immediate precursors. As seen in Table III, the marked CFU-E increase measured *in vitro* was accompanied *in vivo* by the appearance in the spleen of a sizable cohort of proerythroblasts. If the latter are also capable of forming colonies of erythroblasts *in vitro*, some of the CFU-E increases after Ep injection, and conversely some of the decreases in CFU-E in the post-hypoxic polycythemic mouse, would actually be attributable to changes in the number of proerythroblasts present at the time of obtaining spleen or marrow for culture. This possibility has a bearing on the inter-

tion of *in vitro* colony-forming CFU-E measure of erythroid precursors and res further investigation.

Summary. Injection of endotoxin induced spleens of normal and of polycythemic mice a dose-related increase in CFU-E. In the intact normal mice these precursors developed into erythroblasts and resulted in increases in ^{59}Fe incorporation into erythrocytes. In mice whose endogenous Erythroid Precursor (Ep) was suppressed by polycythemia no differentiation of the precursors took place. The time course of the CFU-E increase after endotoxin suggests proliferation of precursors as the most plausible mechanism.

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Effects of Thiols on Sheep Erythrocytes Resulting in Enhanced Rosette Formation with Human T Lymphocytes (39489)

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Sheep red cells (SRBC) adhere specifically to human T lymphocytes (T-L) to form rosettes (1, 2). Since the cellular interaction is weak, marked variance in the number of rosette forming cells (RFC) in normal human peripheral blood has been reported by different investigators (3-5). Recently we described an improved rosetting technique using SRBC treated with the thiol *S*,2-aminoethylisothiuronium bromide (AET) (6). Interaction of AET-treated SRBC (SRBC-A) with T-L results in prompt formation of large rosettes resistant to mechanical disruption so that higher, reproducible percentages of RFC are detected in isolated peripheral blood lymphocytes (PBL). Since a number of mechanisms could account for this enhanced cellular interaction, studies were undertaken to determine the effects of AET and other compounds on SRBC membranes.

Materials and methods. Chemical modification of SRBC. Sterile sheep blood anticoagulated in Alsever's solution was purchased from Grand Island Biological Co. or obtained from a sheep housed at the Minneapolis Veterans Administration Hospital; blood was used within 2 weeks of bleeding unless otherwise specified. AET and 1,1 dimethylguanidine hydrochloride (DMG) were purchased from Aldrich Biochemicals. Bis(2-guanidinoethyl) disulfide (GED) was synthesized by air oxidation of a slightly alkaline, aqueous AET solution. The melting point (198-199°) and infrared spectrum of the product were identical to values reported for GED (7). Dithiothreitol (DTT) was obtained from Calbiochem, 2-mercaptoethanol (2-ME) from Eastman Biochemicals, and iodoacetamide (IA), *N*-ethylmal-

imide (NEM), and dithiodinitrocinic acid (DTDNA) from Sigma Chemical Co. All reagent solutions were freshly prepared just before use. AET and GED solutions ≥ 0.15 M were prepared in 0.01 M, pH 9.0 Tris-HCl buffer (Tris); lesser concentrations of these compounds and all DTT, 2-ME, and DMG solutions were made up in Tris-buffered saline (TBS). IA, NEM, and DTDNA were dissolved in phosphate-buffered (0.01 M, pH 7.4) saline (PBS). One volume of packed, washed SRBC was incubated (30 min, 37°) with 4 vol of AET, DMG, GED, DTT, or 2-ME at concentrations ranging from 1.5×10^{-5} to 3.0×10^{-1} M and washed thoroughly in RPMI 1640 (RPMI). Aliquots of packed SRBC and SRBC-A were incubated (1 hr, 37°) with equal volumes of IA, NEM, or DTDNA at concentrations of 1.5×10^{-5} to 1.5×10^{-2} M and thrice washed in RPMI. Trypsinization of SRBC and SRBC-A was performed by incubating (37°, 1 hr) a 2 mg/ml solution of recrystallized trypsin (Worthington Biochemicals) with equal volumes of 5% SRBC or SRBC-A suspensions in RPMI. Soybean trypsin inhibitor (Sigma Chemical Co.) was added and the cells were washed repeatedly in saline and once in RPMI. If trypsinized SRBC were to be treated with AET, they were washed only once before addition of AET.

Isolation and rosetting of normal PBL. Human PBL were isolated and rosetted with native and chemically modified SRBC as previously described (6). Rosetting enhancement was calculated as the percentage of PBL rosetting with modified SRBC minus the percentage of rosetting with control SRBC (SRBC incubated in buffer).

Solubilization and electrophoresis of SRBC and SRBC-A stroma. Hemoglobin-depleted SRBC and SRBC-A membranes were prepared by the method of Dodge *et*

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solubilized in 1% sodium dodecyl SDS), and electrophoresed in 5.6% lamide gels containing SDS (SDS-9). Gels were stained with Coomassie or PAS and scanned at 530 and respectively.

Measurement of stromal components. Solubility of membrane protein was measured by a modified Lowry method (10). Membrane lipid was measured as described by (11). Phospholipid phosphorus was measured after chloroform:methanol extraction of SRBC and SRBC-A membranes by washing of the solubilized lipids (13), and active (free) and totally solubilized membrane sulfhydryls (SH) were measured with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Chemical Co.) (15, 16). All SH measurements were performed within 2 hours of membrane preparation.

Electron spin resonance (ESR) measurements. These studies were performed in collaboration with Dr. Ronald Mason. All reagents were purchased from Syva. SRBC and SRBC-A were labeled with [1-¹⁴C]stearic acid (5NSA) by addition of 0.1 M 5NSA in ethanol to 0.5 ml of washed erythrocyte suspension. Labeled cells were prepared by incubating red cells with 5×10^{-4} M *N*-(1,6,6-tetramethyl-4-piperidyl) iodide (ISL) or with *N*-(1-oxyl-4-tetramethyl-4-piperidyl) maleimide (L) in PBS. ESR spectra were measured at room temperature.

Effects of various reagents on rosetting. Treatment of SRBC with 0.143 M HCl 9.0, did not alter the protein content, the sialic acid:protein or phospholipid:protein ratios of SRBC-A compared to SRBC (Table I), but markedly enhanced the rosetting activity of these cells. As reported by others (17), the lipid content of SRBC (and of SRBC-A) diminished with storage of blood at 4°C. Compared with SRBC, SRBC-A had a modest (~10%), but statistically significant, decrements in both total ($P < 0.05$) and reactive ($P < 0.001$) membrane phospholipids.

Enhancement of rosetting by AET was due to neutralization of the negative charge of SRBC membranes by

TABLE I. COMPARISON OF MEMBRANE COMPOSITIONS OF SRBC AND SRBC-A

Sheep blood	Storage days at 4°	Protein × 100 dry wt of stroma		SH (10 ⁻⁴ M/mg) protein		Reactive		Sialic Acid (10 ⁻⁴ M/mg of protein)		Phospholipid (μM/mg of protein)	
		SRBC	SRBC-A	SRBC	SRBC-A	SRBC	SRBC-A	SRBC	SRBC-A	SRBC	SRBC-A
VA	1	53.3	52.1	5.96 ± 0.41*	5.86 ± 0.39	2.77 ± 0.21	2.43 ± 0.15	7.77 ± 0.32	8.24 ± 0.35	0.87 ± 0.05	0.74 ± 0.02
GIBCO	1									0.77 ± 0.1	0.80 ± 0.1
GIBCO	6									0.78 ± 0.3	0.80 ± 0.03
GIBCO	10	50.4	49.8	6.20 ± 0.14	5.88 ± 0.19	2.77 ± 0.09	2.49 ± 0.07	7.05 ± 0.25	7.15 ± 0.28	0.77 ± 0.03	0.82 ± 0.03
VA	20	54.7	54.4	6.16 ± 0.16	5.82 ± 0.2	2.79 ± 0.14	2.44 ± 0.11	6.60 ± 0.26	6.64 ± 0.3	0.85 ± 0.08	0.84 ± 0.04
GIBCO	>50	54.2		6.07 ± 0.3	5.65	2.66 ± 0.16	2.39	6.00 ± 0.17	5.79		

* One standard deviation from the mean of four to five individual observations.

strongly cationic guanidino groups of AET, SRBC were exposed to GED and DMG, positively charged, guanidino-containing compounds that lack an active SH group present in AET at pH 9.0. SRBC were also treated with 2-ME and DTT, uncharged thiols, to observe their effects on rosetting. The results (Fig. 1) show that GED and DMG were inactive, but appropriate concentrations of DTT and 2-ME clearly facilitated rosetting. This suggested that SRBC membrane SH groups were required for rosetting. However, treatment of SRBC and SRBC-A with the SH-alkylating agents IA, NEM, and DTDNA at high concentrations ($1.5 \times 10^{-2} M$), before or after thiol treatment, did not impair rosetting; in contrast, trypsinization of native or thiol-treated SRBC totally inhibited rosetting (Table II).

Page-SDS and ESR studies. No discernible differences between SRBC and SRBC-A membranes were detectable by SDS-PAGE or by ESR.

Discussion. The physicochemical forces responsible for adherence of SRBC to T-L have not been elucidated. It has been proposed that coulombic forces or hydrogen bonding between lymphocyte membrane NH_2 groups and negative sites on SRBC are involved (18). Others have suggested that

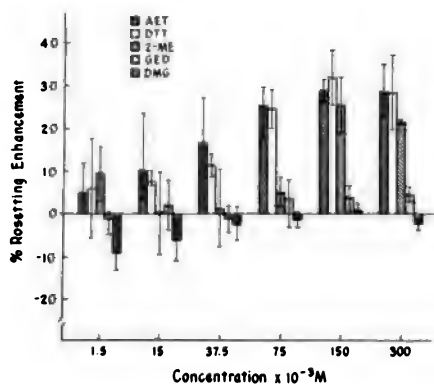


FIG. 1. Dose-response relationship between SRBC treated with various thiols and their analogs to enhancement of rosetting. The mean and absolute variations observed are plotted for each concentration of each compound. Rosetting enhancement is calculated as the percentage of rosetted lymphocytes after exposure to treated SRBC – the percentage of rosetted lymphocytes after incubation with SRBC exposed to buffer alone.

TABLE II. EFFECTS OF SULFHYDRYL INHIBITORS AND TRYPSIN ON SRBC AND SRBC-A ROSETTING

Treatment of SRBC		Percentage of rosetted lymphocytes
Initial	Final	
PBS	PBS	45.8 ± 5.8*
PBS	TBS	38.0 ± 1.0
IA	TBS	40.6 ± 1.0
IA	AET	75.5 ± 4.5
AET	PBS	70.0 ± 4.0
AET	IA	70.8 ± 4.0
NEM	TBS	37.3 ± 4.0
NEM	AET	68.5 ± 1.5
DTDNA	TBS	32.7 ± 4.1
DTDNA	AET	71.5 ± 4.0
Trypsin	TBS	0
Trypsin	AET	0
AET	Trypsin	0

* One standard deviation from the mean of four to five individual observations.

T-L rosetting is mediated by SRBC surface glycopeptides having the oligosaccharide sequence sialic acid → galactose → *N*-acetylglucosamine linked to a mannose:*N*-acetylglucosamine core (19). Since trypsinization removes much of the surface glycoprotein from red cells (17) and abolishes rosetting of SRBC with T-L (1, 2), this supports the concept that SRBC surface glycoprotein serves as the T-L receptor; however, these observations shed no light on the mechanism of thiol-enhanced facilitation of rosetting.

At pH 9.0, AET is converted to 2-mercaptoethylguanidine (20), which contains one free SH group. This thiol readily forms mixed disulfides (S-S) with free SH groups of SRBC membranes. Although many activities of intact erythrocytes cease after they are exposed to SH-active agents, Godin and Schrier (21) have shown that the effects of these compounds may not result simply from membrane SH modification, but rather from structural changes induced in red cell membranes (e.g., solubilization of membrane components or disruption of membrane S-S bridges).

The data (Tables I and II) indicate that free membrane SH groups are not directly involved in rosetting and that thiol-enhanced rosetting is not mediated by mechanisms involving solubilization or removal of SRBC membrane components such as sialic acid, as previously described by Weiner and

associates (22). It appears likely that a major effect of AET and other thiols on SRBC is to disrupt S-S bridges, resulting in important conformational changes in the SRBC membrane. Thiol treatment is known to induce striking immunologic alterations in normal human red cells similar to those observed in paroxysmal nocturnal hemoglobinuria (PNH) erythrocytes (23-25). Several investigators have speculated that cleavage of red cell membrane S-S bonds induces the PNH-like lesion (24, 26-28). We propose that analogous changes in thiol-treated SRBC membranes are responsible for their increased adherence to T-L. However, neither naturally occurring PNH red cells nor normal human red cells treated with AET rosette with T-L (unpublished observations); consequently, thiol treatment of red cells does not appear to create membrane sites that interact nonspecifically with T-L.

If changes in membrane configuration are responsible for thiol-enhanced rosetting, three possibilities must be considered: Thiols may (1) not change the number of reactive sites on SRBC but may stabilize the interaction of exposed sites with T-L; (2) uncover cryptic additional sites identical to those readily accessible on SRBC; and (3) create different, possibly charge-dependent, sites on SRBC membranes. Number 3 is unlikely since various thiols having different charge characteristics are effective in facilitating rosetting of SRBC but not of human red cells; moreover, trypsinization destroys the reactive moiety of thiol-treated, as well as of unmodified, SRBC. Number 2 is improbable since trypsinization destroys all T-L receptor sites on intact SRBC despite subsequent thiol treatment. If buried, potentially reactive sites identical to those accessible on unaltered SRBC membranes exist and are exposed by thiol treatment, it is unlikely that a large molecule such as trypsin (mol wt ~ 20,000) (29) would have access to and destroy such moieties in intact SRBC. The most plausible explanation for thiol-enhanced rosetting is an alteration in SRBC membrane configuration that stabilizes the interaction of surface-reactive groups (probably glycoproteins) with T-L. Unfortunately, electrophoretic and ESR studies did not detect the postulated config-

urational changes. Possibly other techniques, such as fluorescence polarization, may reveal such alterations and, thereby, provide valuable insights into red cell membrane structure and its modification by thiols.

Summary. The mechanism of enhanced interaction of T-L with SRBC-A has been explored and the following results obtained: (1) Compared with SRBC, SRBC-A contain identical quantities of protein, phospholipid, and sialic acid, but have slightly reduced SH content; (2) treatment of SRBC with thiols having charge properties different from AET also facilitates rosetting; (3) exposure of SRBC to various SH-alkylating reagents, either before or after thiol treatment, does not diminish thiol-enhanced rosetting; (4) trypsinization of SRBC and of SRBC-A completely abolishes the ability of these cells to rosette; and (5) no differences could be detected between intact SRBC and SRBC-A, or in their solubilized membrane components, by SDS-PAGE and by ESR. These results indicate that free SH groups of SRBC membranes are not directly involved in the rosetting process. Thiol-enhanced rosetting probably results from cleavage of membrane S-S bridges resulting in configurational changes within the SRBC membrane. The interaction of an SRBC membrane receptor, probably glycoprotein in nature, with T-L is thereby facilitated.

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Immunologic Suppression of DNA Synthesis in MOPC 104E Plasmacytoma Cells (39490)¹

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Lymphocytes respond to various mitogenic antigens by undergoing transformation—a response characterized by increased protein and DNA synthesis followed by cell proliferation (1-3). This type of response can be either augmented or depressed by antibody (4, 5) and the ability of lymphocytes to respond to antigenic stimulation mediated by an immunoglobulin surface receptor (6). For example, Oppenheim found that antigen-antibody complexes (Ab-Ag) either stimulated or suppressed lymphocyte transformation depending on whether antigen or antibody was in excess (4). Banks (5) demonstrated that the cellular response of ovalbumin-sensitive lymphocytes was completely depressed when OA was mixed with anti-OA antisera at concentrations of equivalence and two times antigen with one antiserum and 50 times antigen with another antiserum. Other studies varying from antigen excess to antibody excess resulted in suppression or stimulation of lymphocyte transformation, respectively. Antibody, therefore, is capable of inhibiting the immune response, through a peripheral mechanism (action of antibody on potentially immunoresponsive cells of antigen) or through a central mechanism (effect of antibody on antibody-secreting cells or their precursors) (7). Immunoglobulin can also regulate the immune response, as shown by Sell and others. They demonstrated that anti-immunoglobulin reagents stimulate lymphocyte transformation, and Fanger *et al.* (9)

provided evidence that cross-linkage was required in the stimulation of transformation in rabbit peripheral lymphocytes by anti-globulin reagents. Fab and Fc fragments of goat antirabbit Ig were ineffective in the stimulation of transformation but (Fab')₂ fragments had stimulatory activity comparable to that of the intact antibody. This work supports that of Woodruff (10), who showed that univalent fragments do not cause transformation of human lymphocytes. Recently, Theis (11) demonstrated suppression of delayed hypersensitivity reactions in chickens by passive administration of anti-IgG and anti-IgM, and Herschowitz (12) showed that anti-IgG serum suppressed the anamnestic response of rabbit lymph node cells *in vitro*. In these studies, anti-IgM treatment did not interfere with antibody formation.

Zatz and Goldstein (13) demonstrated suppression of DNA synthesis in mouse spleen after intravenous administration of sheep erythrocytes, *Salmonella typhi* H, or keyhole limpet hemocyanin (KLH). Depression of DNA synthesis was observed as early as 3 hr after antigen stimulation.

The present studies concern the effect of monospecific goat anti- μ antiserum and a bacterial dextran antigen on the incorporation of tritiated thymidine (³HTdR), as a measure of DNA synthesis, by the MOPC 104E myeloma cells *in vitro*. This bacterial dextran has been demonstrated to have specificity for the IgM synthesized by the MOPC-104E tumor cell (14).

Materials and Methods. Antigen. Dextran, fraction S, from *Leuconostoc mesenteroides* NRRL B-1355 was a generous gift of Dr. Allene Jeanes.

Preparation and purification of IgM. IgM was obtained from ascites fluid pool of MOPC 104E tumor maintained by serial

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passage of tumor cells into 6-week-old, female Balb/c mice and purified as follows. MOPC 104E ascites fluid was passed through a column of B-1355 dextran conjugated to Sepharose 2B (15). The bound IgM was subsequently eluted from the column with 0.1 M glycine-HCl buffer, pH 2.8, followed by simultaneous neutralization with borate-saline buffer. The column was also washed with 0.1 N HCl to elute any strongly bound IgM. This technique was possible because the IgM is directed toward certain linkages of bacterial dextran B-1355 and, therefore, reacts specifically with bacterial dextran (14).

The tumor. The MOPC 104E plasmacytoma was obtained through the courtesy of Dr. Michael Potter of the National Institutes of Health. The ascites form of the IgM-producing tumor has since been maintained by serial passage of tumor cells into the peritoneal cavity of normal, 6-week-old, female Balb/c mice (Lab Supply).

Preparation of monospecific goat anti- μ . An immunoabsorbent column of purified MOPC 104E IgM was prepared by coupling to CNBr-activated Sepharose 2B. Goat antimouse IgM serum was passed through the column and the column was washed free of unbound proteins with 0.1 M borate-saline buffer, pH 8.6. Absorbed antibody was then eluted from the IgM column with 0.1 M glycine-HCl buffer, pH 2.8. The eluates were then passed through a λ -light chain column to resolve the anti- μ and anti- λ antibodies (16).

Tumor cell cultures. Ascites fluid was collected by means of a 2.5-cc plastic syringe from the peritoneal cavity of female Balb/c mice bearing 10-day ascitic MOPC 104E plasmacytoma. The ascites fluid was centrifuged at 300g for 5 min in an International Clinical Centrifuge. The supernatant was removed by aspiration and the cells were resuspended in 1 ml of Spinner Modified Medium (BBL) with 20% fetal calf serum (Gibco) and 0.1% penicillin-streptomycin. The cell suspension was diluted with 20% fetal calf serum and viability and cell counts were made in a hemocytometer with trypan blue. The cell suspension was then adjusted to 10^6 tumor cells/ml to serve as stock cell suspension. Samples were then prepared by

adding the desired amount of stock cell suspension (0.7 ml) to 16 \times 125-mm plastic incubation tubes with screw caps (Falcon Plastics) to obtain a final cell concentration of 10^5 cells/cc. After addition of antigen or antiserum to the appropriate concentration, the samples were mixed well and allowed to stand in ice for 0.5 hr. Subsequent to adding medium to attain the final concentration desired, tritiated thymidine (New England Nuclear, sp act 6.7 mCi/mmol) was added to each culture to a final concentration of 2.68 μ Ci/ml of medium. Assay for tritium incorporation was made immediately and the samples were placed on a multipurpose rotator (Scientific Industries, Inc., Model 150V) at 37°. Samples were then taken every 2 hr, up to 10 hr, and assayed for 3 HTdR uptake. Viability was also determined at each interval by the trypan blue exclusion technique.

Differential counts. Differential counts were made in order to determine the relative number of tumor cells and other nucleated cells in the stock cell suspension. Cell dilutions were made to a final concentration of 10^4 nucleated cells/ml (0.3 ml) and sedimented onto microscope slides in a cytocentrifuge. The slides were stained with Wright's stain and the tumor cells were counted. Based on the total number of nucleated cells and the percentage of tumor cells, the actual number of tumor cells was calculated.

The plasmacytoma cells are easily distinguished from other cells in the ascites fluid. The nucleus exhibits indentation and lobulation; the cytoplasm is strongly basophilic. Many cells show appearance of plasma cells or plasma blasts, with considerable variation in size and with occasional binucleate forms. Metastatic figures are frequent.

Nucleophore filter technique for uptake of tritiated thymidine. These experiments were based on the nucleopore technique of Evans and Norman (17) with a modification of Gaudin *et al.* (18) which utilizes a counting solution to lyse the cells prior to filtering and washing. Incorporation of 3 HTdR was measured by means of a liquid scintillation counter in counts per minute (cpm).

Dextran-conjugated SRBC for the detection of complement (19). Radial hemolysis

plates was performed to compare the complement lysing effect of dextran-IgM complexes in the presence of normal fetal serum and guinea pig complement, respectively. Essentially, this method involved coating of the dextran (Fraction S from *L. rooides* NRRL B-1355) (20) with complement and coupling this reaction product to red blood cells. Purified IgM from MOPC 104E tumor (20 μ l) was added to each well of the plate made with the complement-conjugated SRBC in agarose. The plates were then placed in the refrigerator at 4° for 2 hr to allow the IgM to diffuse out of the agarose. Twenty microliters of guinea pig serum or other medium components were added to individual wells. The plates were incubated for 2 hr at 37° and photographed to record evidence of cell lysis.

Effect of Dextran B-1355 on 3 HTdR Uptake. Dextran B-1355 was shown to inhibit the uptake of tritiated thymidine (3 HTdR) in MOPC 104E tumor cells. Tumor cell suspensions (10^5 cells/cc) were incubated at 37° with dextran antigen for 2 hr. The tubes were rotated in an incubator at 37° for 8–10 hr. Antigen concentration varied from 10^{-5} to 10^{-1} mg/ml (excess to excess antibody). Inhibition effect was observed within the range of 10^{-3} mg/ml of antigen. Figure 1 shows the inhibition of 3 HTdR uptake by the MOPC 104E cell suspension. The inhibitory effect became obvious after 2 hr of incubation; there was little further uptake of 3 HTdR in the antigen-containing samples above 10^{-3} mg/ml subsequent to that time. Conversely, at 10^{-4} mg/ml there was no inhibition of 3 HTdR uptake.

Effect of anti- μ on 3 HTdR Incorporation. The effect of anti- μ on 3 HTdR incorporation in MOPC 104E tumor cell suspensions is shown in Fig. 2. The experimental conditions were the same as in the dextran antigen experiment above. Note that there was no inhibition of 3 HTdR uptake after a 2 hr incubation for 10^{-1} and 10^{-3} mg/ml of samples. A concentration of 10^{-5} mg/ml of anti- μ caused no inhibition of 3 HTdR uptake, whereas a continued uptake of 3 HTdR was observed at that of the control sample. The inhibition caused suppression of 3 HTdR incorporation in the concentration range of 10^{-3} mg/ml, similar to the results observed with the dextran antigen.

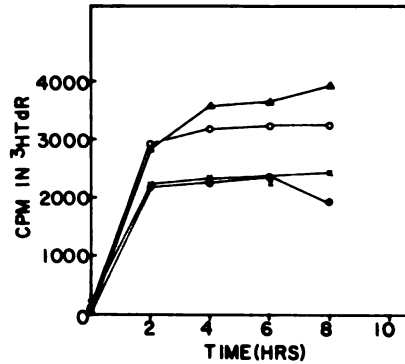


FIG. 1. Inhibition of tritiated thymidine incorporation by dextran B1355 antigen in 80% MOPC 104E tumor cell suspensions (cpm vs time in hr). Each point in time represents one determination of cpm/ 10^5 cells. ○, 10^{-1} mg/ml of dextran B512, control; ×, 10^{-1} mg/ml of dextran B-1355; ●, 10^{-3} mg/ml of dextran B-1355; △, 10^{-4} mg/ml of dextran B-1355.

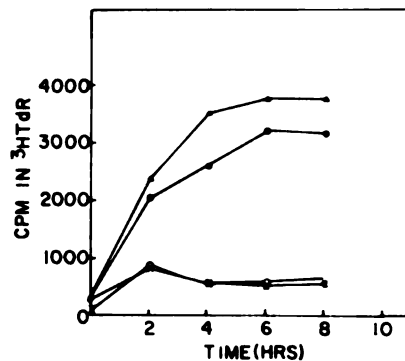


FIG. 2. Inhibition of tritiated thymidine incorporation by monospecific antiserum in 80% MOPC 104E tumor cell suspensions (cpm vs time in hr). Each point in time represents one determination of cpm/ 10^5 cells. ●, control; ×, 10^{-1} mg/ml of anti- μ ; ○, 10^{-3} mg/ml of anti- μ ; ▲, 10^{-5} mg/ml of anti- μ .

Viability by the trypan blue exclusion technique was 80% after 8–10 hr of incubation. The inhibitory effects are likely not due to viability differences, since the viability of the control as well as of the antigen-containing samples were approximately the same.

These same experiments were also run using less homogenous tumor cell populations (12–20% tumor cells based on the total nucleated cells). The results of these experiments were consistent with those reported in this paper, although the relative inhibition using less homogeneous tumors

was slightly less pronounced in the case of the anti- μ experiments. The dextran experiment on less homogeneous cells was comparable to the experiment reported here in terms of the percentage of inhibition of tritiated thymidine uptake.

In view of the demonstrated reproducibility of these experiments, the data reported in this paper were not pooled and thus standard deviations were not reported. Figures 1 and 2 each represent a single experiment. Each point on the curve represents one determination of cpm/ 10^5 cells.

Future experiments utilizing this system as a model will circumvent this problem of obtaining homogeneous tumor cell populations by first separating the tumor cells on a Ficoll gradient prior to incubation with antigen (21).

The possibility existed that the inhibition could be caused by complement cytotoxicity if complement were present in the fetal calf serum. Dextran-IgM complexes have been previously shown to fix complement in this tumor system (15). Therefore, the radial hemolysis technique was utilized to examine this possibility. The results led to the conclusion that the cell suspensions did not contain complement.

Discussion. The present studies demonstrated that both dextran B-1355 antigen and monospecific goat anti- μ inhibit $^3\text{HTdR}$ incorporation in suspensions of MOPC 104E tumor cells. This inhibition could not be attributed to viability differences and was shown not to be the result of complement cytotoxicity.

The results of the antigen and antisera studies indicate that both dextran B-1355 and monospecific anti- μ are capable of suppressing DNA synthesis in MOPC 104E tumor cells *in vitro*. The specific dextran antigen might be expected to stimulate G_0 or G_1 tumor cells to enter the S phase more quickly, or monospecific anti- μ might inhibit entry of G_0 cells into the S phase by blocking effects. Whatever the mechanism, either effect would be interesting and would be reflected by the *in vitro* incorporation of $^3\text{HTdR}$. The inhibitory effect was especially interesting since this system would serve as a model for control of neoplastic growth.

A similar inhibitory effect by monospe-

cific antiserum was also noted. This is of considerable interest in view of the potential of blocking antibodies in transplantation therapy (22). The tumors used in these studies contained a wide distribution of cell types (RBC, polymorphonucleocytes, macrophages, lymphocytes, etc., as well as tumor cells). The possibility that the inhibition was due to complement toxicity of fetal calf serum was eliminated for the dextran system as demonstrated by the failure to lyse dextran-conjugated SRBC treated with MOPC 104E IgM in the radial hemolysis technique. Furthermore, Takahashi *et al.* (23) provided firm evidence that anti- μ was not cytotoxic to MOPC 104E tumor cells even in the presence of complement. Viability checks assured that the inhibition was not due to viability differences in the control and the antigen-containing samples.

In the case of the dextran experiments, the fact that this inhibition was immunologically specific was demonstrated by the lack of inhibition by the nonspecific dextran B512 in the control sample. Evidence that the MOPC 104E tumor cells have immunoglobulin receptors on their surface has been demonstrated previously using fluorescein-conjugated dextran and dextran-conjugated SRBC for the detection of these tumor cells (24).

The mechanism by which anti- μ and the dextran antigen (B-1355) exert their effects is not clear at this time. Whether the mechanism of inhibition by monospecific antiserum is due to antigenic modulation, blocking effects, or some other mechanism will be determined by future experiments, as will the mechanism of dextran inhibition.

Antigenic modulation, an antibody-induced process resulting in specific and selective removal of antigen (IgM in this case) from the cell surface, could provide a mechanism for antiserum inhibition of cellular $^3\text{HTdR}$ uptake and also provide an explanation for tolerance (23, 25).

The relationship between antigenic modulation and "cap formation" has not been established, but it has been suggested that cap formation might provide the mechanism for the phenomenon of antigenic modulation (26). Whether cap formation occurs with these tumor cells in the presence of

antibody has not been investigated, such studies could provide some evidence linking the phenomenon of antigenic stimulation with cap formation; whether respond to antigen by undergoing synthesis and further cell division, or y become nonresponsive to further lation also need to be investigated.

ould also be interesting to ascertain in phase of the cell cycle antigen and rum inhibition occur. Is the effect di- on the S phase or does it simply in- some mechanism whereby cells are nted from making the G_1/S transition. roprobability that cells were inhibited in i_2 is unlikely since inhibition of $^3\text{HTdR}$ e relative to the control was seen and the time of G_2 + time of mitosis ss than 2.5 hr (unpublished observa- this laboratory). Cells would not have ime to progress from early G_2 into S since G_2 + M + G_1 was found to be 7 hr.

other interesting experiment would be at myeloma-bearing mice with the dex- r antiserum and to see if this treatment l either ameliorate or accelerate h of the tumor. Further experiments ring the use of a mitotic inhibitor could lized to determine in what phase of the cle the inhibition effect is exerted.

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Maintenance and Recovery of the Interferon-Induced Antiviral State (39491)

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The paradoxical increase in interferon production following treatment with metabolic inhibitors has led to the hypothesis that a regulatory protein governs the cellular induction of interferon (1-3). There are, however, few reports of similar observations of the effects of inhibitors on the antiviral activity induced by interferon. Potentiation of the antiviral state (AVS) by actinomycin D has been described in L cells (4) and in chick embryo fibroblasts (CEF) (5). Our present experiments describe the effect of cycloheximide treatment on CEF during the decay of the antiviral state following removal of interferon. The level of AVS in chick embryo fibroblasts is indeed dependent upon the presence of interferon in the culture medium. After removal of interferon, however, inhibition of cell protein synthesis by cycloheximide prevents the decay of AVS. Furthermore, after the AVS induced by interferon in CEF has decreased, treatment of cells with cycloheximide leads to the recovery of apparently lost antiviral activity.

Material and methods. Cells and culture media. Primary chicken embryo cells were prepared by trypsinization of 10- or 11-day-old embryos, seeded in 60-mm plastic Petri dishes ($5 \cdot 10^6$ cells), and incubated overnight at 36° in a humidified atmosphere with 5% CO_2 . Eagle's minimum essential medium (MEM), supplemented with 10 or 2% heated fetal calf serum (FCS), was used as growth or maintenance medium, respectively.

Interferon preparations. Interferon was prepared in confluent "aged" monolayers of CEF (5 days) infected with Sindbis virus (0.1 PFU/cell). After infection, cells were incubated for 30 hr at 37° in serum-free PBS without antibiotics. Medium was then collected, centrifuged at 1000g, treated with 2 N HCL O_4 to a final pH of 2 (48 hr), and centrifuged for 30 min at 1000g; the pH of

the supernatant was then adjusted to pH 7 using 5 N NaOH and this was finally centrifuged at 100,000g for 1 hr. Interferon preparations, as compared to chick standard interferon of the Medical Research Council, exhibited an antiviral activity of 3000 units ml^{-1} .

Highly purified chick interferon (300,000 units ml^{-1}), kindly supplied by Dr. Jungwirth (6), was used for a series of experiments.

Interferon assay. CEF cultures ($5 \cdot 10^6$ cells), in triplicate, were treated with 2 ml of interferon (300 units ml^{-1}) diluted in MEM 2% FCS for 6 or 24 hr according to the experiments. Interferon was removed by three washings with PBS, and cells were infected with Sindbis virus at a m.o.i. of 1 PFU per cell and then incubated for an additional 12 hr at 37° . Cell antiviral state (AVS) was determined by the log inhibition of virus yield 12 hr postinfection compared with untreated cultures. Viral titers were estimated by plaque titration in 60-mm plastic Petri dishes with agarose (0.5%) overlay.

Metabolic inhibitors. Actinomycin D was purchased from the Sigma Chemical Company, St. Louis, Missouri, and cycloheximide was purchased from Calbiochem, Los Angeles, California. Preliminary experiments (unpublished results) have shown that (1) actinomycin D ($1 \mu\text{g ml}^{-1}$) inhibited by more than 90% RNA synthesis in the CEF and blocked completely the induction of antiviral effect of interferon; (2) cycloheximide ($10 \mu\text{g ml}^{-1}$) inhibited 90% of the protein synthesis in the CEF; and (3) this inhibition was reversible after three washings of the cells with PBS, whether or not the cells were pretreated with interferon.

Results. Prevention of cellular loss of AVS in CEF by cycloheximide. As shown in Table I, the constant presence of interferon in the extracellular fluid was necessary for the maintenance of the AVS in CEF. At the

TABLE 1. PREVENTION BY CYCLOHEXIMIDE OF CELLULAR LOSS OF THE ANTIVIRAL STATE IN CEF

retreatment for 6 hr	Treatment for 18 hr before wash and Sindbis challenge	Yield of Sindbis virus ^a (log)		Reduction of Sindbis virus yield (log)	
Expt. 1					
Medium	None	9.27	9.10 ^b	—	— ^b
Interferon ^c	None	5.78	5.36 ^b	3.49	3.74 ^b
Medium	Medium	9.27	9.20 ^b	—	— ^b
Interferon	Interferon	5.68	5.40 ^b	3.59	3.80 ^b
Interferon	Medium	7.67	7.30 ^b	1.60	1.90 ^b
Medium	Cyclo ^d	9.25	9.00 ^b	—	— ^b
Interferon	Cyclo	5.59	5.24 ^b	3.66	3.76 ^b
Expt. 2					
Medium	None	9.64		—	
Interferon	None	5.00		4.64	
Medium	Medium	9.64		—	
Interferon	Interferon	5.48		4.16	
Interferon	Medium	7.60		2.04	
Medium	Cyclo	9.60		—	
Interferon	Cyclo	5.48		4.12	

^a Yield of Sindbis virus (log PFU ml⁻¹) was estimated 12 hr postinfection, m.o.i. = 1 PFU/cell.

^b Infection made in the presence of actinomycin D (1 µg ml⁻¹).

^c Interferon, 300 units ml⁻¹, diluted in MEM 2% FCS. Purified interferon (6) was used in Expt. 2.

^d Cycloheximide, 10 µg ml⁻¹.

Note. Each value is the mean of at least two separate experiments in triplicate.

centration used (300 units ml⁻¹), interferon almost completely inhibited the replication of the challenge virus. The antiviral effect was attained after a 5- to 6-hr treatment. If interferon was kept in the medium for a further 18 hr, the AVS remained stable. When interferon was removed at 6 hr and AVS was measured at 24 hr, antiviral activity determined by the log reduction of virus yield decreased by 2 log as may be seen in Table I. Such a decay was, however, prevented by the addition of cycloheximide 6 hr. These data are compatible with the possibility that at this critical moment cycloheximide blocked the formation of a cellular control protein responsible for the decay of the AVS. In order to prevent the possibility of residual induction of AVS during challenge infection, cells were infected in the presence of 1 µg ml⁻¹ of actinomycin D (Experiment 1b). It should be noted that this addition did not modify the results obtained.

Kinetics of decay of AVS and its restoration by cycloheximide treatment. In further experiments, we explore the effect of cycloheximide treatment during the period of decay of AVS. As shown in Fig. 1, about 5–6 hr after removal of interferon the AVS had rapidly decreased and had almost com-

pletely disappeared within 72–90 hr. Similar decay of the antiviral state has been observed in mouse embryo cells (7). Cycloheximide was added at 18, 32, 48, and 72 hr after interferon removal and maintained for 18 hr. Control cells without interferon were also treated in parallel. The inhibitor was then removed, and the cells were washed three times and then infected in the presence of actinomycin D (1 µg ml⁻¹). As shown in Fig. 1, after exposure to cycloheximide, the cells recovered full or partial antiviral activity depending on the addition time of the inhibitor. Blockage of protein synthesis at 18 hr resulted in full recovery of the initial level of AVS at 36 hr (3.50 log inhibition of Sindbis virus yield). At 36 and 48 hr, similar treatment resulted in the restoration 18 hr later of an antiviral activity which reduced virus yield by 2.40 log and 1.40 log. Treatment of cells by cycloheximide increased interferon activity at 36, 54, and 66 hr resulted in a 1000-, 100-, and 10-fold increase, respectively, as compared to the levels of AVS remaining in cells not exposed to the inhibitor. No effect on the challenge virus yield was observed after reversal of cycloheximide in control cells exposed at parallel time intervals to the inhibitor. Thus, the possibility and efficiency of the rescue of

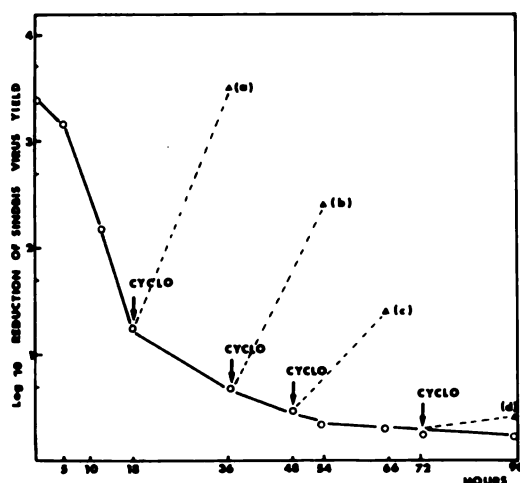


FIG. 1. Time course of decay of the antiviral state and recovery of AVS by cycloheximide in CEF. Induction of the AVS was performed by a 6-hr interferon pretreatment ($300 \text{ units ml}^{-1}$). At 0 time, interferon was removed, and cells were washed with PBS and incubated in MEM 2% FCS. AVS was tested at intervals during a 90-hr observation period (\bigcirc — \bigcirc). Cycloheximide ($10 \mu\text{g ml}^{-1}$) was added for 18 hr at 18, 36, 48, and 72 hr. At 36, 54, 66, and 90 hr, respectively (dotted lines a, b, c, d) inhibitor was eliminated and cells were infected. Infection was made in the presence of actinomycin D ($1 \mu\text{g ml}^{-1}$); AVS is represented by log (PFU ml^{-1}) reduction of yield of Sindbis virus in a 12-hr growth cycle (m.o.i., 1 PFU/cell). Virus yields: in control CEF cells, $1.8 \cdot 10^9$ PFU ml^{-1} ; in control CEF cycloheximide-treated cells, $1.5 \cdot 10^9$ PFU ml^{-1} . Each value is the mean of three experiments in triplicate.

the AVS seemed to depend on the degree of its loss in the interferon controls. When the AVS was completely lost (72–90 hr), treatment by cycloheximide no longer had any effect. Similar results were obtained when highly purified interferon (6) was used.

Effect of cycloheximide treatment on cells treated with increasing concentrations of interferon. The effect of cycloheximide treatment was explored in cells incubated with 7.5, 15, 30, 60, and 300 interferon units for 6 hr (Fig. 2). The antiviral effect was estimated immediately (0 hr), 18 hr, and 36 hr after the removal of interferon. A general decrease in interferon activity was observed at 18 and 36 hr. When cycloheximide was added from 0 to 18 hr, the antiviral effect of the various concentrations tested at 18 hr was maintained at the initial level. When cycloheximide was added from 18 to 36 hr

after the removal of interferon, covered the antiviral activities coming to those initially induced by the interferon concentrations (0 hr). Thus the interferon concentration and the of the AVS remaining at a given time determine the level of AVS which can be covered by an 18-hr exposure of the cycloheximide.

Discussion. The data presented in show that the antiviral state induced by chick interferon decays rapidly when it is removed after a 6-hr treatment; on the contrary, the AVS is completely stable when interferon is in contact with the cells during the whole experiment. This observation indicates that interferon acts continuously on the cells to maintain an otherwise transient antiviral state. It is noteworthy that in CEF cells, after a 6-hr interferon pretreatment, antiviral activity can be maintained if, instead

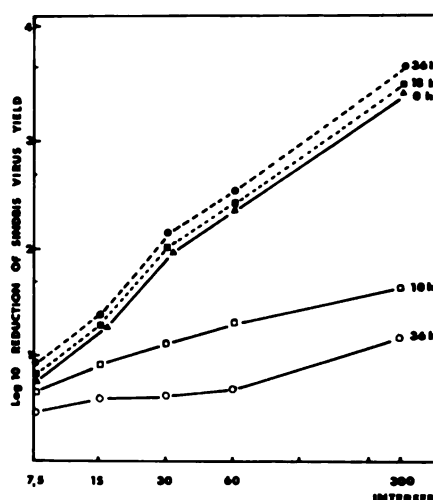


FIG. 2. Maintenance and recovery of the antiviral state by cycloheximide in CEF pretreated for increasing concentrations of interferon. At 0 time, interferon was removed, cells were washed with PBS and incubated in MEM 2% FCS, and AVS was tested immediately (Δ). Subsequently, AVS was estimated at cells incubated in MEM in absence (□—□) or in presence of cycloheximide ($10 \mu\text{g ml}^{-1}$) from 18 hr (■—■) and at 36 hr in cells incubated in absence (\bigcirc — \bigcirc) or in presence of cycloheximide from 18 to 36 hr (●—●). AVS is represented by log (PFU ml^{-1}) reduction of yield of Sindbis virus in a 12-hr growth cycle (m.o.i., 1 PFU/cell). Virus yields: in control CEF cells, $1.2 \cdot 10^9$ PFU ml^{-1} ; in control CEF cycloheximide-treated cells, $1.5 \cdot 10^9$ PFU ml^{-1} .

an, an inhibitor of protein synthesis cycloheximide is added to the cells. results suggest that concomitant to uction process of the antiviral state, a control requiring integrity of protein is promotes the degradation and/or ation of one of the presumed ele-[antiviral protein(s) (AVP) or AVP which mediate the antiviral state.

bserved in Figs. 1 and 2, cyclohexi-reatment, performed during the first of the decay of the antiviral state, s cells to regain their initial antiviral . After 36 hr the possibility of recov-owing treatment by the inhibitor de-gradually and disappears at 90 hr. bservation suggests at first that the control protein we postulated here be subjected to a rapid turnover and reversibly inactivate one of the meta-eps, resulting finally in the antiviral nd more likely (in view of our experi-conditions) the ultimate step, i.e., al protein(s) (AVP). In this case, the ould not be immediately degraded uld first be converted into an inactive n the absence of cell protein synthe- l reactivation could occur during the hr after removal of interferon. After he AVP would be progressively de-and the antiviral state only partially d. Such a mechanism of regulation ng adjustment of the rate of degrada-enzyme activity has been proposed uced tyrosine aminotransferase in rat *vivo* (8).

e the effect of cycloheximide must be d to permit viral multiplication, in o test the antiviral state, new antiviral i(s) may have been rapidly translated nRna(s) which accumulated during cloheximide treatment. A similar ism may possibly account for the su-ction of interferon production fol-treatment by cycloheximide (3). Yet, een found that in chick cells treated rferon, cycloheximide prevented the oment of antiviral activity (9), pre-y by allowing degradation of unsta- RNA (10). The possibility of a new on of mRNA(s) for AVP(s) occurring moval of cycloheximide, during chal-nfection, could also be considered. unlikely in view of the results re-

ported in Table I. Experiment 1b, and Fig. 1. In these experiments, indeed, mainte-nance and recovery of AVS are observed in cells where, after cycloheximide treatment, infection was made in the presence of acti-nomycin D ($1 \mu\text{g ml}^{-1}$) in order to block cellular RNA synthesis. Thus, although di-rect evidence is missing, in our system, cy-cloheximide might act more by preserving the activity of AVP than by increasing the amount of mRNA available for its transla-tion.

Summary. The antiviral state in CEF de-cays rapidly after the removal of interferon. Inhibition of cell protein synthesis by cyclo-heximide prevents this decay. Even after the decay of the antiviral state the addition of cycloheximide to the cells is followed by recovery of antiviral activity. The treatment by cycloheximide does not act by increasing the direct antiviral effect of various concen-trations of interferon but rather by blocking the synthesis of a postulated regulatory pro-tein which could reversibly inactivate the antiviral protein or its expression.

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Diethylnitrosamine-Induced Changes in Mouse Liver Morphology and Function¹ (39492)

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When rats and mice of various strains are exposed to the carcinogens, dimethylnitrosamine (DMN) or diethylnitrosamine (DEN), over prolonged time periods a number of proliferative and neoplastic changes occur in the liver. Parenchymal cell tumors, either hepatocellular carcinoma or hepatoma, have been reported (1-3) usually preceded or accompanied by hyperplastic nodule formation. In other investigations the predominant tumors have been cholangiocarcinomas or hemangiomas (4, 5). Bile duct proliferation to a greater or lesser extent occurs in all rats and mice treated with one of these dialkylnitrosamines (1-3).

Complex interactions occur between the dialkylnitrosamines and drugs which alter the activity of the liver drug-metabolizing enzyme systems (DMES). For example, phenobarbital administration which increases the activity of liver DMES enhances the mutagenic effects of the dialkylnitrosamines (6), but diminishes their neoplastic potential (7). Indeed, the acute administration of DMN or DEN to rats or to mice decreases liver DMES activity (8-10), a seemingly paradoxical effect for chemicals whose activation requires metabolism by these enzyme systems (11, 12). Whether a comparable depression in liver DMES activity accompanies long term administration of low doses of DMN and DEN is not known.

This report describes the effects on hepatic morphology of DEN administration over periods of 10 to 24 weeks to adult, male Swiss mice. Hexobarbital sleeping times and the response to a large dose of carbon tetrachloride have been used as *in vivo* indicators of the status of the liver DMES.

Methods. Adult male Swiss mice (COBS

CD-1, obtained from the Charles River Breeding Laboratories, Inc., Wilmington, Mass.) weighing 25 to 30 g were maintained 5 per cage and permitted constant access to food and water. DEN was added to the drinking water of several groups of mice to provide a concentration of 4 mg/100 ml. Phenobarbital sodium was incorporated in the drinking water of other groups of mice to provide a concentration of 30 mg/100 ml. After 10 days of exposure to phenobarbital, DEN in a concentration of 4 mg/100 ml was additionally added to the drinking water and 21 days later the phenobarbital sodium concentration was increased to 45 mg/100 ml. The reasons for using this dosing schedule for phenobarbital have been described in detail (13).

Hexobarbital sleeping times were determined in control and DEN-treated mice 10 and 24 weeks after the start of the experiments and in mice treated with phenobarbital plus DEN at 10 weeks after the start of the administration of DEN. Mice were killed 3 days after hexobarbital sleeping times had been determined, complete autopsies were performed, and segments of liver were fixed in 10% buffered formaldehyde solution. All sections were stained with hematoxylin eosin and, where appropriate, slides were stained with the Masson trichrome stain and with Wilder's reticulin stain. Frozen sections were stained with oil red O.

A preliminary observation suggested that DEN-treated mice were unusually resistant to the acute effects of CCl₄. The following experiments were performed to substantiate this observation. Fourteen control mice, seven mice treated with DEN for 10 weeks, and eight mice treated with DEN for 24 weeks were given CCl₄ in corn oil by gavage to provide a dose of CCl₄ of 0.5 μ l per gram of body weight. Experience in this laboratory indicates that this dose is approximately

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LD_{50} for CCl_4 in mice which have not received any other drugs or chemicals. Mice were observed for lethality and morbidity a period of 42 hours and the surviving mice were then killed and autopsied, and sections of liver were taken for light microscopy. The malevolent signs associated with morbidity are difficult to describe in detail. However, overt manifestations such as "sick appearance," lordosis, and sluggish responses to normal stimuli were taken as evidence of morbidity. Another indication of CCl_4 effects was the presence of grossly enlarged yellow or cream-colored livers.

Results. Gross changes in the livers of mice treated with DEN for 10 weeks were not remarkable; the livers of DEN-treated mice tended to be firmer than the control livers. In mice given DEN for 24 weeks, the livers generally presented a granular surface, and nodules which were discrete and could be considered grossly to be tumors were observed on 10 of 27 livers from mice given DEN for 24 weeks. In five of these mice, nodules were solitary and very hemorrhagic. There were no tumors in other body tissues examined—lungs, GI tract, kidneys, urinary bladder, pancreas, or brain.

The histologic appearance of liver tissue from mice given DEN for 10 weeks was comparable to that described by Clapp and Craig (3). Some hepatocytes tended to be enlarged with large nuclei containing coarsely stained chromatin. The characteristic change seen in all sections was oval cell proliferation, presumably the result of biliary duct cell hyperplasia. By 12–14 weeks of treatment with DEN, bile duct proliferation and microcyst formation had become prominent and a few scattered necrotic hepatocytes also were present in most sections. Foci of inflammatory cells, mainly lymphocytes, were present in all sections.

The addition of phenobarbital did not basically modify the effects of 10 weeks of exposure to DEN on liver morphology. There appeared to be even more extensive oval cell proliferation in some sections of liver segments taken from mice treated with phenobarbital and DEN when compared with liver tissue from mice treated only with DEN for 10 weeks.

Bile duct proliferation and cyst formation had become widespread, even overwhelming, in livers from mice treated with DEN for 24 weeks (Fig. 1). The hepatic parenchyma often was divided into nodular masses (Fig. 1), but there was scant deposi-

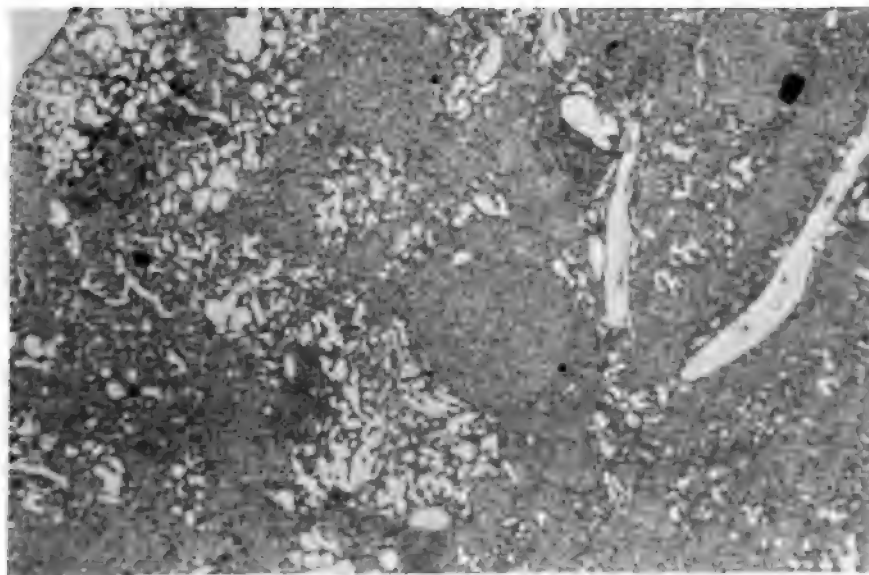


Fig. 1. Liver section from mouse given DEN in the drinking water for 24 weeks, showing extensive proliferation of bile ducts and nodule formation. $\times 25$.

tion of collagen. The granular appearance of the liver, grossly giving an appearance of cirrhosis, resulted from the extensive proliferation of bile ducts and hepatic nodule formation rather than from deposition of fibrous tissue. A number of hepatocytes in each section contained bizarre, enlarged nuclei with prominent nuclear inclusions (Fig. 2). Scattered throughout the sections were foci of inflammatory cells, primarily lymphocytes (Fig. 2).

Many of the tissue nodules were composed of hyperplastic cells and in two sections the cytology of the cells within the nodules resembled hepatomas. No hepatocellular carcinomas were found. The only well-defined neoplastic changes seen were one hemangioma, and in two sections, tissue suggestive of cholangiocarcinoma. The hemorrhagic surface nodules were blood-containing cysts surrounded by proliferating bile ducts.

Hexobarbital sleeping times were significantly increased in mice treated with DEN for 10 weeks (Table I), an effect which was markedly enhanced in mice treated with DEN for 24 weeks (Table I). Hexobarbital sleeping times in mice treated with both DEN and phenobarbital were similar to controls (Table I).

Carbon tetrachloride administration resulted in the death of 6 of 14 control mice within 26 hr after CCl_4 had been given (Table II). Morbidity was observed in each of the remaining eight mice and fatty liver was present in all 14 mice (Table II). Histologically, liver sections from CCl_4 -treated control mice showed the characteristic effects of CCl_4 —extensive central lobular necrosis and widespread fatty change. There were no deaths, morbidity, nor fatty livers in any of the DEN-treated mice given carbon tetrachloride (Table II). None of the characteristic histologic changes of CCl_4 was observed in most sections from DEN-treated mice. The only histologic evidence of some effect of CCl_4 was hepatocytes showing a ballooning-type degeneration, which were scattered throughout the sections of livers taken from four of the mice treated with DEN for 10 weeks.

Discussion. Long-term exposure of non-Swiss mice to DEN produced an adaptive response characterized by biliary duct cell hyperplasia which in later stages resulted in widespread bile duct proliferation. Hepatic parenchymal cells appeared to be much less affected by the nitrosamine, and well-defined evidence of hepatocytic response was not seen until at least 12 weeks.

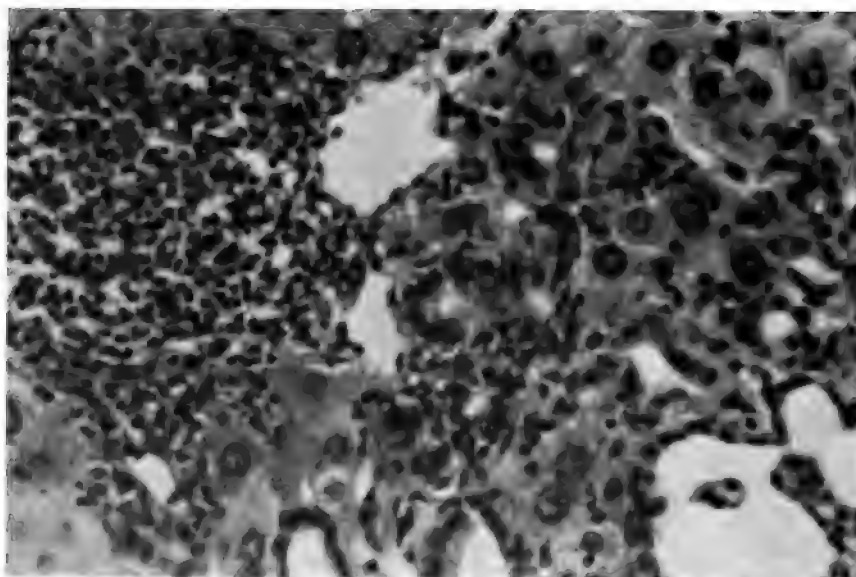


FIG. 2. Higher magnification showing details of bile duct proliferation and an aggregate of lymphocytes. 100.

TABLE I. HEXOBARBITAL SLEEPING TIMES IN CONTROL MICE AND IN MICE TREATED WITH DIETHYLNITROSAMINE (DEN) AND WITH PHENOBARBITAL AND DIETHYLNITROSAMINE (PB-DEN)^a

Group	N ^b	Sleeping times (min)
Control	9	32.4 ± 3.6
DEN (10 weeks)	13	61.2 ± 4.7 ^c
PB-DEN (10 weeks)	8	33.0 ± 3.5
Control	8	30.6 ± 2.1
PB-DEN (24 weeks)	13	160.6 ± 17.4 ^c

The results are expressed as the mean ± one standard error of the mean. Hexobarbital sodium was dissolved in 0.154 M NaCl solution and administered intraperitoneally at a dose of 100 µg/g of body weight. Number of mice in each group.

^c < 0.001.

TABLE II. EFFECT OF AN ACUTE DOSE OF CCl₄ IN CONTROL MICE AND IN MICE TREATED WITH DIETHYLNITROSAMINE (DEN)

Group	Deaths	Morbidity within 42 hr ^a	Fatty liver at 42 hr ^a
Control	6/14	8/8 ^b	8/8 ^b
DEN (10 weeks)	0/7	0/7	0/7
DEN (24 weeks)	0/8	0/8	0/8

^aForty-two hours after the administration of CCl₄ to surviving mice.

exposure to DEN. The effects of DEN on hepatic morphology in this strain of Swiss mice were similar to the responses to DEN in mice of the RF strain (5), although the incidence of neoplastic transformation was low in the Swiss mice. Foci of lymphocytic infiltration were a prominent feature of adaptative responses of the liver to chronic exposure to DEN. Possibly, this indicates a high level of immunosurveillance which may be related to the low incidence of neoplasia.

CCl₄ must be metabolized by liver DMES to more highly reactive derivatives, which are the ultimate hepatotoxins (14-16). The increased resistance by DEN-treated mice to the hepatotoxic effects of CCl₄, therefore, probably reflected a marked diminution in liver DMES activity. If liver DMES activity is markedly reduced by exposure to DEN, it would be logical to assume that DEN-treated mice would show minimal effects to doses of CCl₄ which produced se-

vere hepatotoxicity in untreated mice. The marked increases in hexobarbital sleeping times support the conclusion that DEN progressively decreased liver DMES activity. That there was continued morphologic response to DEN despite the apparent reduction in liver DMES activity suggests either that a minimal level of liver DMES activity was present, sufficient to activate DEN, or that systems other than those associated with the liver microsomes activate the dialkyl nitrosamines (17). The metabolism of exogenous chemicals could also have been altered by the intense, widespread proliferation of bile ducts, but there is no information regarding such influences on drug metabolism and disposition.

The apparent cancellation by phenobarbital of DEN-induced increases in hexobarbital sleeping times could have been the result of simple addition of the stimulation by phenobarbital of liver DMES activity and its depression by DEN. However, phenobarbital treatment generally increased the acute toxicity of potential liver toxins and enhanced some of the effects of long-term administration of hepatotoxins (13). The interaction between phenobarbital and hepatotoxins on liver DMES activity when each is given concurrently over long time periods is unknown. Moreover, adaptative changes in liver ultrastructure and DMES activity occurred during long-term administration of phenobarbital to rats (18), while in some strains of mice, liver cell changes suggestive of neoplastic transformation accompanied chronic phenobarbital administration (19). Although phenobarbital prevented DEN-induced increases in hexobarbital sleeping times, corresponding reduction of DEN-induced morphologic changes at the light microscopic level did not occur.

Summary. Long-term administration of DEN to adult Swiss mice for periods of up to 24 weeks resulted in intense bile duct proliferation, formation of hepatic nodules, some of which appeared to be hyperplastic, and foci of lymphocytic infiltration. No hepatocellular carcinomas or well-defined hepatomas were found in these DEN-treated mice. The only tumors observed were one hemangioma, and in 2 sections, tissue suggestive of cholangiocarcinoma. The sig-

nificant increases in hexobarbital sleeping times and the striking resistance to the hepatotoxic effects of CCl_4 in DEN-treated mice indicated that long-term exposure to DEN resulted in a progressive inhibition of liver DMES activity. Phenobarbital administration concurrent with DEN prevented the decreases in hexobarbital sleeping times but did not alter the morphologic changes in the liver produced by DEN, at least at the light microscopic level.

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Myoelectric Activity of the Small Intestine of the Dog during Total Parenteral Nutrition^{1, 2} (39493)

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Parenteral nutrition (TPN) is used in the management of patients who inadequately assimilate food stuffs in the gastrointestinal (GI) tract (1). Exactly, TPN is useful as a tool to determine the role of total oral food deprivation on the physiology and morphology of the GI tract. Previous studies in several laboratory animals have demonstrated major changes in the structure and physiology during periods of intravenous alimentation. In the dog, the small bowel undergoes hypomotility, brush border enzymes significantly decrease, and serum and antral gastrin concentrations are diminished (1-3). However, little is known of the effects of TPN on the myoelectric activity of the GI tract.

Myoelectric patterns of the small intestine can be examined by studying its myoelectric activity which consists of two basic types of waves: slow waves, or basic electric rhythm (BER), and spike potentials. Descriptions of these two potentials and their relationship to controlling contractions of the bowel have been reported (4). Basically, slow waves control the timing of contractions (they occur) at any one site and at all sites, while spike potentials signal the onset of the contractions. Spikes appear during a specific phase of the cycle; however, not all slow waves are accompanied by spike potentials. The percentage of slow waves that are accompanied by spike potentials and their distribution depend on the diges-

tive state of the animal. In the fasted dog, a characteristic pattern called the intestinal interdigestive myoelectric complex is present (5). This complex consists of four phases. In phase I, none of the slow waves is accompanied by spike potentials. In phase II, spike potentials are superimposed on the slow waves in a random fashion. During phase III, every slow wave has superimposed spike potentials. Phase III ends abruptly (phase IV) and phase I repeats. These phases not only occur at one particular site, but appear to propagate aborally along the bowel. Feeding the dog abolishes this complex and produces a more uniform distribution of spike potentials superimposed upon slow waves, which has been called the fed pattern of activity. The exact percentage of slow waves with spike potentials depends on the type of food given and on the region of bowel being monitored (6). The purpose of this study was to determine the intestinal motility patterns in dogs maintained by TPN.

Materials and Methods. Three dogs, two male and one female, were used. Each animal was anesthetized with thiopental sodium (20 mg/kg) and methoxyflurane. A laparotomy was done, and 14 electrodes were sewn onto the serosal surface of the small intestine. The monopolar, silver-wire electrodes were spaced equidistant (about 25 cm apart) along the entire bowel from the gastroduodenal junction to the ileocecal junction. A coil of silver wire was placed subcutaneously in the right flank to serve as a reference electrode. Details for constructing and implanting similar electrodes have been reported (7). During the same operation, a sterile Polyvinyl catheter (i.d. 0.03 in.) was placed in the superior vena cava through a cut down in the external jugular

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of the interdigestive myoelectric complex during the short-term fasted periods between oral nourishment is not known. Code (10) has suggested that this complex could serve as an "interdigestive housekeeper" which maintains the small intestine free of accumulated secretions and ingested debris. Possibly, this fasted activity may prevent bacterial overgrowth within the small intestine.

Castro *et al.* have demonstrated that several changes occur in the small intestine of the rat during periods of at least 6 days of TPN (2). A change in the diameter of the small intestine during TPN has also been observed in the dog (personal observation). Although hypoplasia of the small intestine may occur during nutritional maintenance with TPN, the myoelectric activity of the small bowel did not change. Similarly, the elicitation of a fed pattern of motility immediately following the ingestion of food indicated that the mechanism responsible for conversion from the fasted pattern was not impaired during TPN. The factors responsible for conversion of the fasted to the fed pattern following eating are unknown. The fact that the fed pattern does not occur during TPN indicates that the presence of nutrients in the blood is not responsible for the conversion. Thus, events which occur due to the actual presence of food in the GI tract must be important. Release of the hormone gastrin has been implicated in the conversion (11). Since serum and antral gastrin concentrations are thought to be depressed during long intervals of "bowel rest," as occurs with TPN (12), gastrin may not have been available to participate in the conversion from fasted to fed patterns in our experiment.

The capacity of an animal to assimilate food immediately following a prolonged period of TPN has been questioned because several investigators have demonstrated that brush-border hydrolytic enzymes are depressed during nutritional maintenance with TPN (2, 3). Nevertheless, each animal in our experiment ate canned dog food without ill effects immediately after TPN was terminated.

Summary. The myoelectric activity of the small intestine was monitored in conscious

dogs before, during, and after peri total parenteral nutrition (TPN). In TPN, each animal displayed two distinct patterns of myoelectric activity. One pattern was seen in an animal that had been fasted for 18 hr (the interdigestive myoelectric complex); the other pattern was seen during feeding (fed pattern). TPN was then discontinued and continued for up to 11 weeks, at which time the animals maintained weight. During TPN, the only myoelectric pattern evident was the interdigestive myoelectric complex and its characteristics were similar to those seen before TPN. After first oral feeding after TPN was followed by the development of a fed pattern. We conclude that: (1) The fasted pattern of myoelectric activity is present during prolonged periods of food deprivation. (2) The presence of nutrients in the blood is not a factor required for presence of the fed pattern. (3) The factors responsible for development of the fed pattern are not impaired by oral food deprivation.

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Kinetics of Erythroid Cell Precursors in the Newborn Rat¹ (39494)

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The cell-cycle time of erythroid cells in animals has been estimated by a number of investigators (1-3) but there are a few studies of the cell-cycle time of recognizable erythroid precursors in rats.

In the newborn rat during the first week there is a striking increase of the erythropoiesis in the bone marrow that at this time significant numbers of erythroid precursor cells similar to those seen in the adult liver during the hepatic phase of erythropoiesis. These cells have a mean diameter of about 30-40 μ m, a pale basophilic cytoplasm, a leptochromatic nucleus with one or two nucleoli, and frequently occur in

the newborn animal matures, these undifferentiated cells gradually decline in frequency and there is also a gradual change in the functional response of the bone marrow to experimental perturbations, known to be able to abolish erythropoiesis in the adult rat as bilateral nephrectomy, hyperoxia, or iron induced plethora, starvation, hypoxic polycythemia (4). It has been reported that CFU's derived from fetal liver promote an earlier recovery of erythropoiesis in lethally irradiated animals than a comparable number of marrow CFU's, suggesting a difference in the generation time of fetal liver erythroid precursor cells and, by inference, of the narrow erythroid precursors during the first weeks of life, when erythropoiesis is proceeding at the fastest rate (5). The results of the work to be reported in this paper, on the cell-cycle time of the recognizable erythroid precursors in 5-, 10-, and 20-day-old rats was measured by the technique of labeled mitoses.

Materials and Methods. Newborn rats of

the Sprague-Dawley strain, aged 5, 10, and 20 days, and 10- to 14-week-old female rats of the same strain weighing 180-200 g were given a single intraperitoneal injection of [methyl-³H] thymidine (³H-Tdr) (1.0 μ Ci per g of body weight, specific activity 10 Ci/mole).

One to three rats were sacrificed from each age group at intervals thereafter. Three to seven animals were used for each experimental point for a total of 235 newborn and 48 adult rats in three separate studies.

The smears were prepared from bone marrow, air-dried, and fixed in methyl alcohol for 3 min.

Autoradiographs were prepared by the dipping technique, using Kodak Nuclear Track Emulsion, type NTB 3, diluted 1:1 with distilled water.

After a suitable exposure time (21 days), the slides were developed with Kodak D19 developer at 19°C for 2 min, rinsed in 0.5% acetic acid, stop bath, fixed for 2 min in Kodak's Unifix, rinsed for 2 hr in running water, and then soaked for 1 hr in a buffer solution at pH 6.3.

The slides were stained with Wright's and Giemsa solutions.

The percentage of erythroid mitoses was determined by counting at least 100 mitoses on each preparation.

A mitosis was considered as labeled if more than three grains could be attributed to the chromosomes.

Approximate values for the cell-cycle parameters were obtained from the labeled mitoses curve (FLM) by a graphic method (6).

The experimental data were analyzed using the method of Steel and Hanes (7) for computer simulation of FLM curves.

Results. The cell-cycle is typically subdivided into the following phases: M, mitotic phase; S, DNA synthetic phase; G₁, postmitotic presynthetic phase; and G₂, postsynthetic premitotic phase. The method of la-

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beled mitoses is one of the most important methods for studying and estimating the intermitotic intervals and the length and position of the S period. It consists of pulse-labeling the cells with tritiated thymidine and observing the proportion of mitoses which are labeled (FLM) at a series of subsequent times after injection of the label (6).

The FLM method is based on the assumption that all cells in the S phase at the time of the injection of the tracer will be labeled, and that no others will take up label.

A theoretical curve for a synchronous population in which all cells have no variation of the time spent in the different parts of the cycle would have a rectangular shape, and the duration of the nuclear cycle and its phases could be estimated from such data.

However, it is clear from the available

experimental data obtained by the technique of the FLM that all cell populations have a considerable variability in the timing of the phases of the cycle, therefore the curves show a greater or lesser degree of damping, in which case, approximate estimates for the mean values of the cell parameters can be obtained.

Figures 1 and 2 show the FLM curves for pro- and basophilic erythroblasts in 5- and 20-day-old and adult rats.

The points represent the mean of the seven experimental points and the lines are the curves calculated by the computer.

The usual method of estimating the cell cycle time from a FLM curve is to measure the interval between the first and the second ascending limb of the curve (6).

Inspection of Figs. 1 and 2 reveals

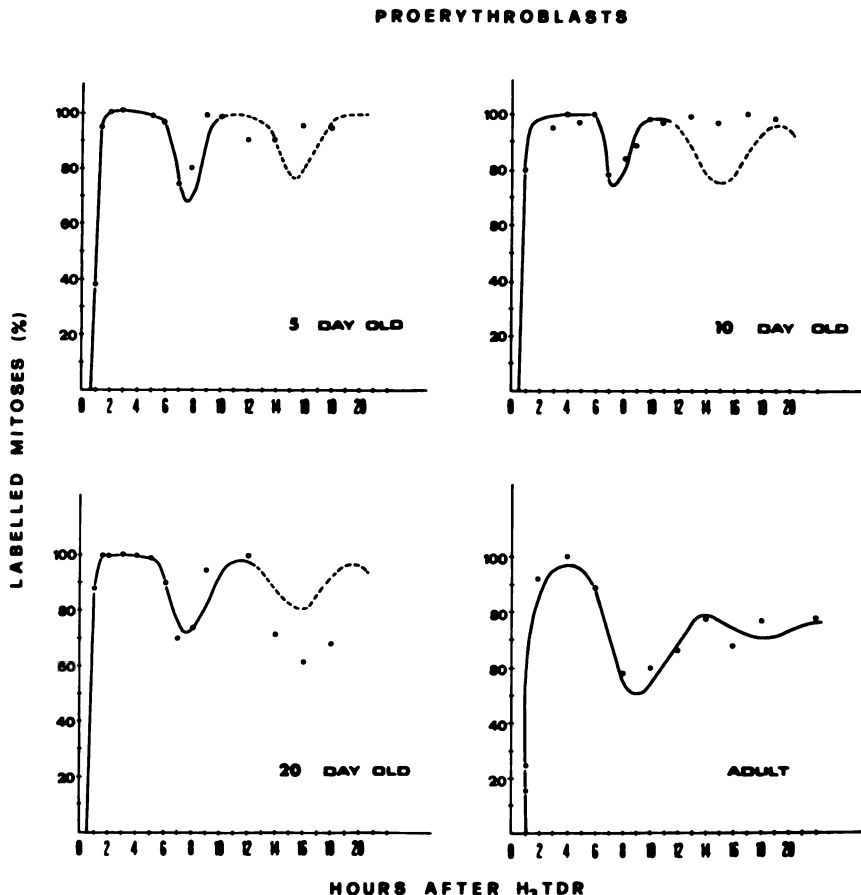


FIG. 1. Labeled mitoses curves for proerythroblasts in 5-, 10-, and 20-day-old and adult rats, at various times after injection of 3H -thymidine. Each point represents the mean of three to seven animals. The curves drawn are calculated by computer analysis of the data.

BAS. ERYTHROBLASTS

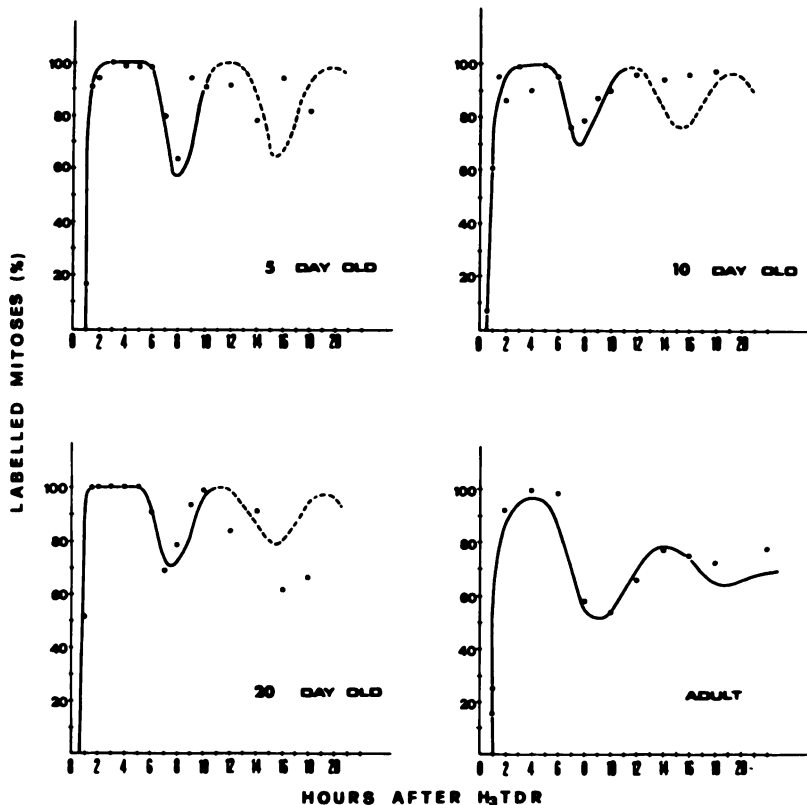


FIG. 2. Labeled mitoses curves for basophilic erythroblasts in 5-, 10-, and 20-day-old and adult rats, at various times after injection of ^3H -thymidine. Each point represents the mean of three to seven animals. The curves drawn were calculated by computer analysis of the data.

the mean generation time in the adult rat is about 10 hr, and in the neonatal rats the cycle time is about 7.5 hr.

The first labeled mitotic figures appear between 30 and 45 min after tritiated thymidine administration, thus, the premitotic rest period (G_2) is about 0.75 hr. The duration of DNA synthesis is 5.50 hr and this leaves $7.5 - (5.5 + 0.75) = 1.25$ for $G_1 + M$.

Although it is easy to get approximate values for the cell-cycle parameters from the FLM curve, there is no graphical method which has any degree of precision.

A more detailed analysis of the labeled mitoses curves has been obtained using the method of Steel and Hanes (7), a development of the method of Barrett (8) in which theoretical curves are computed on the basis of a model which assumes a log-normal distribution of the duration of G_1 , S, and G_2

phases; the analysis consists in finding a labeled mitoses curve which is the best fit to the experimental data. For the purpose of this analysis, the duration of mitosis (M) is divided equally between G_1 and G_2 .

The curves fitted to the experimental points in Figs. 1 and 2 are computed curves, obtained by Steel's method.

The computed curves do not fit the experimental data adequately over a whole cell cycle (broken curves), especially for the newborn rats, but the computer was able to generate curves to fit the first wave of labeled mitoses in each case.

The analysis should therefore be restricted to the first cycle, which is sufficient to give reasonable estimates of the cell-cycle parameters; the means, standard deviations, and medians of these parameters are shown in Table I.

The values obtained for the cycle times by

Steel's method are in good agreement with those estimated by simple inspection of the curves.

The durations of both G_1 and G_2 for the earlier erythroblasts of the newborn rats are always shorter than those of the adult, but S

is not appreciably different from the adult.

For the basophilic erythroblasts, the reduction of G_1 and G_2 is associated with a slight reduction of S (from 7.5 to 6.6–6.9, respectively).

The analysis of the corresponding distributions of cell-cycle times shown in Fig. 3 demonstrates a considerable decrease of spread in the cell-cycle in the newborn rats.

Discussion. The labeling mitoses curves represent the resultants of the time intervals for the replicating cell populations.

In the present study, the FLM curves for the newborn rats show that the descending limb falls only to 60–70% and then rises again to 95–98%.

This may be due to the overlapping of groups of rapidly dividing cells so that the "in phase" pattern is lost, the lengthening of the second cell-cycle as a result of the radiation effect of $^3\text{H-Tdr}$ (9), and the entry into erythroid compartment of labeled cells from an actively proliferating precursor compartment.

Reutilization of tritium may also be playing some part because erythropoiesis is very active and more erythroid cells are losing their initially labeled nuclei during maturation, possibly making more DNA available for reutilization (10).

However, had any of the above mentioned events occurred then the FLM curves would have given an overestimate of the generation time.

The estimates obtained by Steel's method show that in the newborn rats considerable

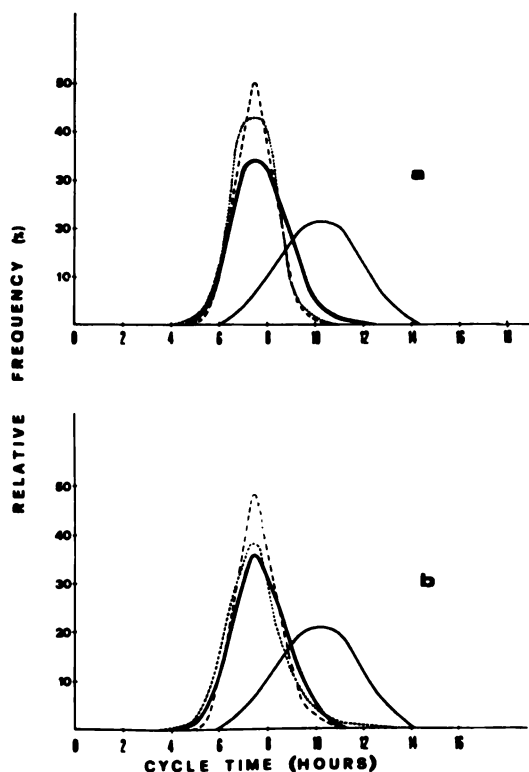


FIG. 3. The distribution of cell-cycle times in 5(-----), 10(.....), 20(—) day-old and adult (—) rats. a: Proerythroblasts; b: Basophilic erythroblasts.

TABLE I. MEAN VALUES OF THE CELL-CYCLE PARAMETERS IN 5-, 10-, AND 20-DAY-OLD AND ADULT RATS*

Rats (days old)	Cell type							
	Proerythroblast				Basophilic erythroblast			
	$G_1 + 0.5 M$ (hr)	S (hr)	$G_2 + 0.5 M$ (hr)	Median t_c (hr)	$G_1 + 0.5 M$ (hr)	S (hr)	$G_2 + 0.5 M$ (hr)	Median t_c (hr)
5	0.6 ± 0.3 (0.5)	6.9 ± 0.7 (6.9)	0.1 ± 0.1 (0.05)	(7.5)	1.0 ± 0.4 (0.9)	6.6 ± 0.6 (6.6)	0.1 ± 0.3 (0.03)	(7.6)
10	0.4 ± 0.6 (0.2)	6.8 ± 0.7 (6.7)	0.2 ± 0.1 (0.07)	(7.3)	0.8 ± 0.6 (0.6)	6.7 ± 0.9 (6.7)	0.1 ± 0.1 (0.09)	(7.5)
20	0.5 ± 0.2 (0.5)	7.1 ± 1.1 (7.0)	0.3 ± 0.1 (0.2)	(7.8)	0.5 ± 0.2 (0.5)	7.0 ± 1.0 (6.9)	0.3 ± 0.2 (0.2)	(7.7)
Adults	1.9 ± 1.4 (1.5)	7.0 ± 1.8 (6.7)	1.2 ± 1.6 (0.7)	(9.8)	1.4 ± 1.5 (1.1)	7.9 ± 2.4 (7.5)	1.8 ± 1.0 (1.6)	(10.7)

* Mean values were derived by Steel's method. The median values are in parentheses; for a log-normal distribution, median = $\left[\text{Mean} / \left\{ 1 + \left(\frac{\text{Standard deviation}}{\text{Mean}} \right)^2 \right\} \right]^{1/2}$.

is occur in the cell-cycle time of the erythroid precursors, and that reductions would seem mainly due to changes in the duration of G_1 and G_2

Results reported here are in fair agreement with those estimated by Roylance (12), who observed distinct shortened cell-cycle time of erythroid precursor response to anemia in bled and drazine-treated adult rats.

It has been extensively documented that, at birth, there is hypochromic anemia and from this one may infer that the shortened generation time of erythroid cells in the newborn rats are due to action of erythropoietin associated with hypochromic anemia.

Further, the erythropoietic activity removed from the plasma of newborn rats exposed to hypoxia was significantly less than observed in adults that were similarly exposed, indicating a low rate of erythroid synthesis in early neonatal life.

The number of differences has been reviewed between fetal and adult murine

the thymidine suicide technique, *et al.* (14) found no depression of normal adult marrow, but found killing of CFU's with cells from marrow. This suggested that few CFU's in marrow are in cell-cycle, while a high proportion of CFU's in fetal liver may be in cell-cycle. Kubanek *et al.* (15) tested differentiation of adult marrow and fetal CFU's, using secondary transplants after transplantation of adult marrow, and a 3-4 day lag phase followed by sequential growth of splenic CFU's. In slices of fetal liver tissue this lag period was significantly diminished and the exposure to growth had a faster doubling time, giving a shorter generation time of fetal CFU's.

Shorter doubling time of fetal liver marrow was also found by Schofield (17). The shift from hepatic to myeloid erythropoiesis begins in rodents in late fetal life. Neonatal rat is in transition from adult erythropoiesis (4).

From our results it would appear that the generation time of erythroid elements in early neonatal life is shorter than that observed for adult erythroid elements.

Whether the explanation of such a difference resides in a different stem cell, similar to that present in the fetal liver, is moot, but it is tempting to suggest that this is the case.

Summary. The kinetics of erythroid proliferation have been studied in the newborn rat of different ages by the method of ^3H -Tdr-labeled mitoses. The experimental results have been analyzed by the method of Steel and Hanes (7).

A generation time of 10 hr has been obtained in the adult rats for the proerythroblasts and basophilic erythroblasts, while in the newborn rat the generation time for the same cells was found to be of the order of 7.5 hr. It has been shown that these reductions are mainly due to decrease in the duration of G_1 and G_2 phases.

The possibility is suggested that the shorter cell-cycle of the erythroid cells could, at least in part, account for the erythroid hyperplasia observed in the rat during the first 2 weeks of life.

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Studies on the Relationship of Parathyroid Hormone and Calcitonin to Plasma and Liver Phosphate¹ (39495)

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Parathyroid hormone and calcitonin, with vitamin D, are considered to be regulating hormones. However, all three influence phosphate homeostasis, although by different mechanisms. Parathyroid hormone increases renal excretion of phosphate (1), vitamin D increases intestinal absorption of this ion (2), and calcitonin decreases its transport out of plasma (3). The interrelationship of the three is thought to specify the plasma phosphate concentration. No "feedback" system related to phosphate has been identified. Other hormones affect plasma phosphate. Two of the most important are insulin and glucagon. Following the administration of either, plasma phosphate concentrations fall. Following the administration of glucose, plasma phosphate concentrations fall. Following the administration of glucose, plasma phosphate concentrations fall.

It has been known for many years that plasma phosphate levels in the laboratory rat fluctuate during the daylight hours, only to reverse the daily fluctuations of this ion at night (4). One of the interrelationships of this study was the finding that in the normal rat, plasma calcium and phosphate fluctuated in the same direction during the daily cycle, except that the plasma phosphate were considerably

lower. It was observed that calcitonin decreases plasma phosphate by moving this ion from the ECF (3), attempts have been made to determine into which tissue this ion was moved. While some phosphate is lost in urine following calcitonin administration, this loss is not considered to account for decreases in plasma phosphate (7). Recently, it has been found that liver inorganic phosphate is decreased by parathyroid hormone and increased by calcitonin (8, 9).

This study was carried out to examine in greater detail the relationship of parathyroid hormone and calcitonin to the daily fluctuation in plasma phosphate and to investigate the possible involvement of the liver in these events.

Materials and methods. Holtzman rats weighing between 150-260 g were used for these experiments. The phosphate-deficient diet was obtained from Nutritional Biochemical Co. It contained less than 0.15% P. Most animals were maintained on standard Purina Laboratory Chow under 12-hr light and 12-hr dark conditions in which food was available during the dark period.

All surgery was performed under light (ether) anesthesia. Parathyroid glands, when autotransplanted, were individually placed laterally in the hyoid muscle. After surgery, rats were allowed a minimum of 3 days for recovery, extended to 2 weeks following gland transplantation. The day before rats were used experimentally, a blood sample was obtained and plasma calcium was analyzed to determine adequacy of surgical procedures. Thyroidectomized (TX) rats were maintained on T₄ (50 µg/kg three times weekly).

The calcitonin used was purified salmon calcitonin (SCT) furnished by Armour Pharmaceutical Co. and assayed at 400 MRC U/mg. Parathyroid hormone was a TCA powder obtained from Inolex Pharmaceutical Division and assayed at 179 U/mg.

Sacrifice was by decapitation to permit rapid blood drainage. The central (ventral) lobe of the liver was rapidly removed, frozen in liquid nitrogen, and transferred in dry ice to a freezer maintained at -40°C.

For phosphate analysis, a procedure modified from Wahler and Wollenberger (10) was utilized. The lobe of liver was weighed, frozen, homogenized in 0.5 N perchloric acid (PCA), centrifuged at 0°C, and the supernatant was kept on ice for the remain-

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der of the procedure. An aliquot was immediately shaken with 0.015 M sodium molybdate in isopropyl acetate. After separation, an excess of stannous chloride was added to the upper phase which was analyzed for inorganic phosphate. A separate aliquot of the PCA supernatant was hydrolyzed at 70°C for at least 2 days in sealed ampules and analyzed for inorganic phosphate colorimetrically by the method of Chen *et al.* (11). The resultant data were recorded as milligrams of inorganic phosphate and milligrams of organically bound acid soluble phosphate per gram wet weight of liver. The latter value (organically bound) was obtained by subtracting the value for inorganic phosphate from the total acid soluble phosphate value.

Results. 1. Daily changes in plasma phosphate concentrations. Plasma phosphate concentrations rise during the daily fasting portion of the feeding cycle in the rat and fall abruptly during the feeding portion (6). These daily changes were reported to be less marked in thyroidectomized rats bearing functional parathyroid glands (TX). In the present study, data were obtained for plasma phosphate levels in thyroparathyroidectomized (TPTX) rats and further observations were made in TX rats. These data are summarized in Fig. 1.

In the absence of the parathyroids, plasma phosphate concentrations are elevated. However, the daily cyclic nature of these concentrations is retained. Plasma phosphate levels, as in normal rats, reach their maximum near the end of the fasting portion of the daily cycle and fall rapidly during feeding (Fig. 1A).

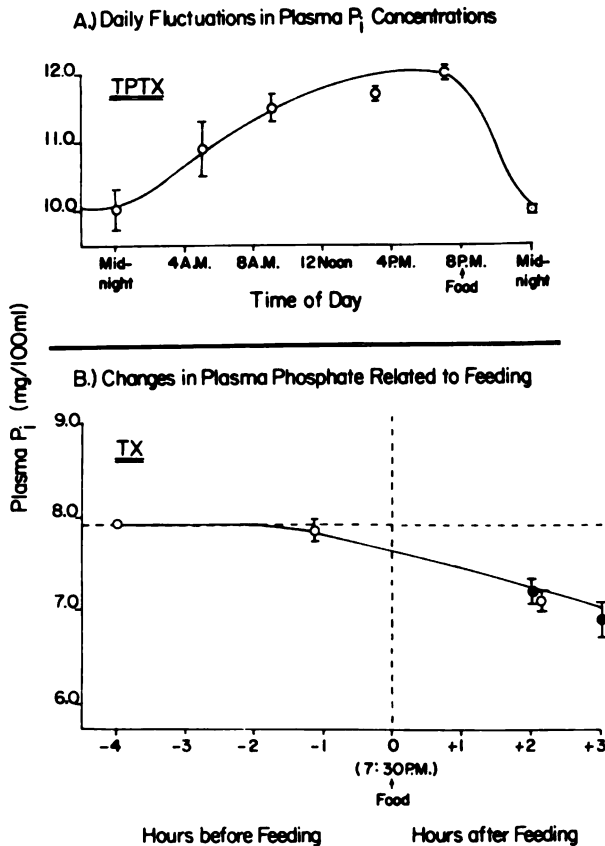
Since one of the purposes of this study was to relate calcitonin function to phosphate homeostasis, changes in plasma phosphate concentrations in TX rats were reexamined. The study focused on that period of the day just prior to, and for a few hours after the rats were provided food. In normal rats conditioned to a regular feeding schedule, plasma phosphate concentrations start their fall several hours prior to feeding and continue to fall during the first few hours of feeding, even if, on any one day, food is withheld for a few hours (12). The same pattern can be seen in the data obtained

from TX rats (Fig. 1B). While the changes are less marked than in normal rats, plasma phosphate values fall during the fed portion of the daily cycle. It also occurred in rats fasted for several additional hours.

It can be concluded from these results that, while both hormones, affect plasma phosphate concentrations, the daily cyclic nature of these concentrations is due at least in part to other causes.

2. The effect of extended fasting on plasma PO_4 changes. In these experiments three groups were studied: normal rats, rats maintained for 2–3 weeks on a phosphate deficient diet, and rats TPTXed for 10 days. In each group, food was removed from half the animals late in the evening. Plasma phosphate concentrations in plasma from blood samples taken in the evening were compared to those taken the next morning. It should be borne in mind that after an extended period of time on a phosphate deficient diet, the parathyroid glands atrophy and are no longer functional (13). The data are summarized in Table I. In each of the three groups, comparison is made between fasted and fed conditions. In normal rats, the early morning plasma phosphate values were the same whether or not the rats had been fed or fasted during the night hours. In both the TPTX group and that on a low PO_4 diet the fasted values in the morning were higher than the corresponding fed values. These results emphasize the reciprocal relationship of plasma phosphate changes to digestive processes.

3. The effect of feeding versus fasting on liver phosphate concentrations. For these experiments, the acid-soluble fraction of liver phosphate was divided by the procedures described above into inorganic phosphate and that organically bound. Plasma phosphate values at time of sacrifice are given in micromoles for ease of comparison to liver concentrations. The data are summarized in Table II. In each experiment, the rats were weighed the afternoon prior to separation into "fed" and "fasted" groups and were divided such that the average body weight of those to be "fasted" or "fed" was not significantly different. Three groups were studied: normal rats, TPTX-rats, and rats on the low PO_4 diet.



Plasma phosphate changes in thyroidectomized (TX) and in thyroparathyroidectomized (TPTX) rats: (1) were maintained on a 12-hr light, 12-hr dark schedule. Food was provided at the time indicated for the 1 hr. Availability of food was not necessarily correlated with either the light or dark period. (2) All represent mean of five to 15 different plasma samples \pm SE. (3) In 1 B, \circ = nonfed rats; \bullet = fed rats. \circ after represents a single experiment when availability of food was delayed 2 hr. (4) All plasma samples were from rats maintained on a food and light schedule for two or more weeks.

At weight of the lobe of liver was approximately 30% greater in the "fasted" condition in all instances this appeared to be too great a difference to be accounted for solely by the loss of liver glycogen, a separate experiment was done to determine how much of the weight was due to fluid incorporated into liver. These data are given in Table 1. The percentage of water was relatively constant, indicating both no organic addition or loss in the two

regardless of this difference in liver weight, the inorganic phosphate values for "fasted" were greater than for "fed" when expressed as P_i per gram wet weight. How-

ever, when expressed as P_i per lobe of liver, all values were surprisingly constant regardless of diet or group. Only in the normal rats was there any significant increase in the inorganic phosphate concentration per lobe of liver due to feeding.

For the acid-soluble organically bound phosphate pool, values are given only as total bound phosphate/lobe of liver. The phosphate concentration was much higher in the "fed" condition in all groups.

The particular points of interest concerning these data to our present study are: (1) The inorganic phosphate/lobe of liver was constant regardless of the plasma PO_4 concentration at the time of sacrifice and regardless of whether the rats had been fed or

TABLE I. EFFECT OF OVERNIGHT FASTING ON PLASMA PHOSPHATE CONCENTRATIONS

Time sample obtained	Plasma phosphate (mg P/100 ml)	
	10 PM ^a	9 AM
Controls		
(a) Fed	> 9.05 ± 0.16 (17) ^b	8.52 ± 0.21 (8)
(b) Fasted		8.06 ± 0.17 (8)
Low PO ₄ diet		
(a) Fed	> 3.88 ± 0.28 (6)	3.50 ± 0.11 (17)
(b) Fasted		6.90 ± 0.14 (11) ^Δ
TPTX		
(a) Fed	> 14.59 ± 0.30 (20)	12.1 ± 0.25 (20)
(b) Fasted		14.45 ± 0.19 (22) ^Δ

^a Blood sample taken prior to removal of food from cages.^b Values = mean ± SE with number of animals utilized in parentheses.^Δ Significantly different from "fed" rats of same group with $P < 0.01$.

TABLE II. LIVER PHOSPHATE CONCENTRATIONS: FED VERSUS FASTED ANIMALS

	Body wt (g) ^a	Liver wt (mg)	Plasma P _i (μM/ ml)	Liver P _i		
				μM/g wet wt	μM/lobe	μM/Lobe of liver ASB ^b
1. Normals						
Fed (8) ^c	165 ± 3.7	988 ± 52	2.7 ± 0.07	6.54 ± 0.23	6.49 ± 0.33	21.81 ± 1.32
Fasted (8)	167 ± 4.3	699 ± 34 ^Δ	2.6 ± 0.05	7.59 ± 0.27 ^Δ	5.31 ± 0.33 ^Δ	15.64 ± 0.89 ^Δ
2. TPTX						
Fed (9)	152 ± 4	880 ± 33	4.0 ± 0.1	5.93 ± 0.13	5.22 ± 0.23	21.46 ± 0.77
Fasted (9)	149 ± 3	603 ± 20 ^Δ	4.6 ± 0.1 ^Δ	9.28 ± 0.45 ^Δ	5.48 ± 0.22	15.76 ± 0.56 ^Δ
3. Low PO ₄ diet						
Fed (11)	177 ± 4	1072 ± 52	1.16 ± 0.04	5.22 ± 0.30	5.86 ± 0.47	28.59 ± 1.97
Fasted (11)	174 ± 4	782 ± 24 ^Δ	2.23 ± 0.05 ^Δ	8.20 ± 0.24 ^Δ	6.38 ± 0.21	19.89 ± 0.76 ^Δ

^a Animal weight obtained late afternoon prior to separation into "fed" and "fasted" groups. All other values obtained at 9 AM the following day.^b Acid-soluble bound phosphate.^c Numbers in parentheses equal number of animals. Values given ± SE.^Δ Statistically different from "fed" rats of same group with $P < 0.01$.

TABLE IIA. RAT LIVER WEIGHTS (WET WT VS DRY WT)

	Body weight (g)		Liver weights (AM)		
	PM	AM	Wet wt (mg)	Dry wt (mg)	Water (%)
Fed (3) ^a	289 ± 4.7	286 ± 4.2	1276 ± 29	385 ± 8	70.9 ± 1.2
Fasted (3)	290 ± 4.2	274 ± 4.9	966 ± 30 ^Δ	288 ± 14 ^Δ	69.8 ± 0.1

^a Numbers in parentheses equal number of animals.^Δ Statistically different from "fed" rats run concurrently, with $P < 0.01$.

fasted; (2) neither TPTX nor a low phosphate diet appeared to exert any significant influence on the increase in the concentrations of phosphate in the acid-soluble organically bound pool which occurred as the result of feeding, or the decrease due to overnight fasting; and (3) the inorganic phosphate concentration of liver when compared on the basis of micromolar per gram wet weight was always greater than that found in plasma.

4. *Liver phosphate concentrations in "fed" rats as influenced by the absence of the thyroids.* Since a previous study (12) had determined that the actual entrance of food

into the digestive tract was a major cause for the secretion of calcitonin, these experiments were done to determine whether the presence of calcitonin increased the uptake of phosphate by liver during a single feeding period. The data are summarized in Table III. Three groups of rats were compared: normal versus TPTX; control rats (bearing parathyroid transplants and with active thyroid glands) versus TX rats with parathyroid transplants; and TPTX versus PTX rats. The only significantly different values were in plasma phosphate levels at time of sacrifice, as was expected from the treatment. Neither the absence of the thyroids or para-

thyroids appeared to influence either the liver inorganic phosphate or the uptake of phosphate by the liver as the result of overnight feeding.

5. *The influence of exogenous hormones on liver phosphate.* Since it has recently been reported (8, 9) that both PTH and calcitonin influence liver inorganic phosphate, a series of experiments was run in which these two hormones were injected singly or in combination to TPTX rats fed overnight. In the first experiment, four hourly injections of the hormones were made before the rats were sacrificed and liver samples were analyzed. In the second experiment, the time was extended to 8 hr. The data are summarized in Table IV.

It can be noted that plasma phosphate fell as expected following hormone treatment and was maintained at the lower levels

throughout the experiment (see column 4 of Table IVA and B). The inorganic phosphate component of liver (per lobe) was also lower in all three experimental groups at the 4-hr post-hormone time period (Table IVA). However, by 8 hr, despite continued hormone treatment, liver inorganic phosphate values all had returned to their normal range (Table IVB). While PTH appeared to cause a slight drop in the acid soluble organically bound component, this difference was not statistically significant.

Discussion. These and earlier studies have demonstrated that plasma phosphate concentrations drop as the result of anticipation of feeding and actual entrance of food into the digestive tract. Conversely, plasma phosphate concentrations rise during extended fasting. This rise is prevented by the presence of an actively secreting parathy-

TABLE III. LIVER PHOSPHATE CONCENTRATIONS IN THE ABSENCE OF THE THYROID

	Body weight ^a (g)	Liver weight (mg)	Plasma P _i (μM/ml)	Liver P _i (μM/lobe)	Liver ASB ^b (mm/lobe)
Normal (5) ^c	167 ± 11.5	879 ± 112.42	2.51 ± 0.06	6.98 ± 0.74	21.46 ± 2.74
TPTX (6)	161 ± 6.9	967 ± 87.67	3.95 ± 0.09 ^d	7.56 ± 0.45	22.63 ± 1.89
PTT (15)	264 ± 3.56	1437 ± 57.27	3.29 ± 0.07 ^d	7.09 ± 0.39	29.44 ± 0.88
TX (14)	264 ± 4.04	1335 ± 39.38	3.29 ± 0.06 ^d	6.10 ± 0.25	28.05 ± 0.85
PTX (12)	227 ± 6.2	1148 ± 48.7	4.37 ± 0.11 ^d	6.06 ± 0.29	27.00 ± 0.94
TPTX (10)	212 ± 7.0	1035 ± 45.9	4.31 ± 0.08 ^d	5.37 ± 0.22	24.49 ± 1.17

^a Animal weight obtained late afternoon prior to the availability of food. All other values obtained at 9 AM the following day.

^b Acid-soluble bound phosphate.

^c Numbers in parentheses equal number of animals. Values given ± SE. TPTX = thyroparathyroidectomized. PTT = thyroidectomized with parathyroid transplants. TX = thyroidectomized. PTX = parathyroidectomized.

^d Statistically different from normal rats, with $P < 0.01$.

TABLE IV. LIVER PHOSPHATE CONCENTRATIONS FOLLOWING HORMONE ADMINISTRATION

	Body wt ^a (g)	Liver wt (mg)	Plasma P _i (μM/ml)	Liver P _i (μM/lobe)	Liver ASB (μM/lobe)
A. Injected hourly for 4 hr					
Veh. (12) ^b	172 ± 3.7	870 ± 29.5	4.13 ± 0.16	5.54 ± 0.26	19.9 ± 0.96
SCT (13) ^c	175 ± 4.5	896 ± 27.6	3.26 ± 0.09 ^d	4.94 ± 0.19 ^d	20.9 ± 1.22
PTH (6) ^d	179 ± 5.4	886 ± 29.4	2.49 ± 0.04 ^d	4.55 ± 0.43 ^d	18.6 ± 1.55
Comb. (7) ^e	167 ± 6.0	827 ± 43.0	2.13 ± 0.10 ^d	4.50 ± 0.16 ^d	20.9 ± 1.23
B. Injected hourly for 8 hr					
Veh. (12)	179 ± 5.7	848 ± 50.04	3.57 ± 0.14	5.08 ± 0.40	20.42 ± 1.60
PTH (6)	180 ± 3.0	843 ± 17.80	2.14 ± 0.18 ^d	5.62 ± 0.32	19.79 ± 1.43
Comb. (7)	180 ± 8.7	859 ± 51.30	1.25 ± 0.13 ^d	5.05 ± 0.19	21.17 ± 1.90

^a Animal weight taken late afternoon, all other values the following day. All values given ± SE.

^b Veh. = vehicle injected controls.

^c SCT dose = 0.2 mU/g/hr.

^d PTH dose = 0.1 U/g/hr.

^e Comb. = both hormones injected together.

^d Statistically significant from vehicle injected controls run concurrently, with $P < 0.01$.

^d Statistically significant from vehicle injected controls run concurrently, with $P < 0.05$.

roid gland and conversely is most dramatically illustrated in rats maintained several weeks on a phosphate deficient diet and then fasted overnight. In the fasted condition, the plasma phosphate concentration of the phosphate deficient rats almost doubles, rising into the low normal range for this species. This suggests that the phosphate changes may be secondary to other metabolic events.

The difference between the size of the acid-soluble organically bound phosphate pool in the livers of fed and fasted rats also supports the conclusion that phosphate changes may largely be the result of other metabolic events. These liver values are influenced markedly by digestive activity but not by a phosphate deficient diet or by plasma phosphate concentrations varying from 1 to 4 $\mu\text{M}/\text{ml}$.

The inorganic phosphate concentrations of liver remain relatively constant and unaffected by feeding or fasting. These levels were higher than that in plasma, even in thyroparathyroidectomized rats, and were not statistically reduced after 3 weeks on a phosphate deficient diet. Abrupt changes in plasma phosphate by hormone injection tended to cause a temporary drop in liver inorganic phosphate, but these returned to normal even under continued hormone administration, which produced even lower plasma phosphate levels. It appears, therefore, that the liver inorganic phosphate pool is under some type of homeostatic control.

The primary purpose of this study was to relate parathyroid hormone and calcitonin action to these phosphate changes. It is concluded that neither hormone directly affects liver phosphate. No evidence was obtained to support our hypothesis that the hypophosphatemic action of calcitonin could be attributed at least in part to its ability to move phosphate from plasma to liver. However, movement into other soft tissue or bone has not been ruled out. The discrepancy between these results with CT and those reported earlier (8, 9) may be accounted for by the previous use of only fasted TPTX rats. CT injection in such rats may lead to severe tetany. Any rats in tetany were discarded from our experiments, since such rats tended to have variable and

usually elevated phosphate values. Neither PTH or CT appears to be a primary causative agent in the plasma phosphate drop occurring at the onset of feeding, since it occurred in the absence of both hormones. The role of insulin or other intestinal-related hormones remains yet to be determined, being beyond the scope of this project.

While these data do not suggest any role for PTH on plasma phosphate other than its action on renal phosphate excretion, they do raise the question as to why plasma phosphate values continue to rise throughout the fasting segment of the daily feeding cycle. If feeding is delayed, extending the fasting period, plasma phosphate values are maintained low as long as PTH is available. It is obvious that while parathyroid hormone sets the plasma phosphate level by its renal action, it is unable to prevent the daily rise during the fasting portion. Yet PTH secretion must occur during the fasting portion of the daily cycle or the plasma calcium concentrations would not be at their highest level (6) at this time.

Summary. The relationships of parathyroid hormone and calcitonin to plasma and liver phosphate concentrations were studied in rats. While parathyroid hormone affected plasma phosphate levels, it did not control the daily fluctuations in rats adapted to standard feeding and light conditions. The endogenous presence of this hormone did prevent the additional rise in plasma phosphate concentrations which occurs in its absence following an overnight fast.

Inorganic phosphate concentrations of liver were always higher than plasma phosphate and were surprisingly constant despite almost fourfold variation in plasma concentrations. They remained relatively constant even 3 weeks after the rats were placed on a low phosphate diet and after extended thyroparathyroidectomy. Only minor differences were seen in liver inorganic phosphate levels in fed and fasted rats. It is concluded that this phosphate pool is under some type of homeostatic control.

The organically bound phosphate in the acid-soluble pool of liver increases rapidly after feeding, decreasing just as rapidly on fasting. These changes were not influenced

by the phosphate level of plasma nor the presence or absence of either endogenous parathyroid hormone or calcitonin and occurred while the rats were on a low phosphate diet. It is concluded that neither parathyroid hormone nor calcitonin directly affects these two acid soluble phosphate pools in liver.

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Effects of Hypoxic Exposure on Embryonic Implantation in Mice¹ (39496)

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A number of studies describe the effects of hypoxia (exposure to low oxygen in gaseous mixtures, reduced oxygen tension in hypobaric chambers, and natural high altitude) on pregnancy during the postimplantation phase of gestation (1-7). Little is known of the effects of varying degrees of hypoxia on reproductive function prior to placentation. Recently, Rattner and Ramm (8) demonstrated that exposure of gravid mice to a 7% oxygen environment (equivalent to the partial pressure of oxygen at 24,000 ft) resulted in death of embryos during the preimplantation period or shortly thereafter, indicating that gestation may be interrupted prior to placentation.

The present investigation was conducted to determine the level of oxygen which impairs blastocyst implantation and to follow the effects of progressively more severe degrees of hypoxia. Morphologic changes in the ovaries, uteri, and embryos were used to elucidate a possible mechanism by which exposure to hypoxia alters gestation.

Materials and Methods. Ten-week-old virgin female mice (strain CD-1), weighing 25-30 g, were mated monogamously. Mating was confirmed by visual inspection for vaginal plugs prior to 1000 hr on the following day. The day on which a vaginal plug was first observed was designated as Day 0 of pregnancy. Gravid mice were assigned to one of five groups and provided with food and water *ad libitum*.

The various oxygen environment exposures were performed in a Labconco fiberglass glovebox (Labconco Co., Kansas City, Mo.) to which ultrahigh purity nitrogen was added to reduce the partial pressure of oxygen (pO_2). A Beckman Model D2 Oxygen Analyzer (Beckman Instruments, Inc., Fullerton, Ca.) was used to monitor pO_2 , and carbon dioxide tension (pCO_2) was measured with a Fyrite Carbon Dioxide Analyzer (Bacharach Industrial Instrument Co., Pittsburgh, Pa.). The pCO_2 within the chamber was reduced by intermittently circulating the atmosphere through a 6 M KOH bubbling flask. Relative humidity was held between 40-70% by means of a $CaSO_4$ drying train. Chamber temperature ranged from 22-25°C and a 14-hr light:10-hr dark photoperiod was employed.

Group (Gp) 1 included mice maintained at $21 \pm 0.5\%$ oxygen (approximate pO_2 , 159 mm Hg and equivalent to sea level) with the carbon dioxide concentration less than $0.5 \pm 0.5\%$. Animals in Gps 2 and 3 were exposed to $14 \pm 0.5\%$ oxygen (approximate pO_2 , 131 mm Hg and equivalent to 10,000 ft) and $12 \pm 0.5\%$ oxygen (approximate pO_2 , 91 mm Hg and equivalent to 14,000 ft), respectively, with the carbon dioxide concentration less than $1.5 \pm 0.5\%$ of total atmospheric constituents. Group 4 consisted of mice maintained in $10 \pm 0.5\%$ oxygen (approximate pO_2 , 76 mm Hg and equivalent to 18,000 ft) with the carbon dioxide concentration $\leq 1.5 \pm 0.5\%$ and, lastly, Gp 5 at $8 \pm 0.5\%$ oxygen (approximate pO_2 , 60 mm Hg and equivalent to 22,000 ft) with the carbon dioxide concentration $\leq 2.0 \pm 0.5\%$. Following insemination, animals were placed into the glovebox and exposed continuously to the various oxygen environments from 1000 hr on Days 0 through 6 of pregnancy. Mice were maintained in an animal care facility ($21 \pm 0.5\%$ oxygen, $\leq 0.5 \pm 0.5\%$ carbon dioxide) until Day 8, at which time they were sacrificed by cervical dislocation.

Body weights were measured before and immediately after chamber exposure and on Day 8 of pregnancy. From the suborbital sinus, 50 μ l of blood was drawn for microhematocrit determination on Days 6 and 8.

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were obtained on uteri and paired and adrenal glands. Generally, the gest corpora lutea (CL) were dissected with a needle from each ovary, blot-dried on filter paper, and rapidly weighed to a sensitivity on a Cahn Model RTL balance (Cahn Instruments, Cerritos, CA). If no implantation sites were present, contents of one uterine horn were placed into a watch glass with 0.5 ml of a 1% NaCl and examined under a dissecting microscope for preimplantation embryos and uterine debris. Tissues were fixed in 10% formalin, sectioned serially at 10 μ m, and stained with hematoxylin and eosin for microscopic analysis.

The number of mice with implantation sites in each treatment group was compared by a chi-square contingency analysis. The data were also tested for homogeneity of variance that utilization of analysis of variance or the Student's *t* test was appropriate. Newman-Keuls multiple range test was employed to determine significant differences between means.

Macroscopic observations. The age of mice with implantation sites decreased with the reduction in oxygen concentrations (Table I). Implantation was not observed at 21 or 14% oxygen concentrations (Groups 1 and 2). At 12 and 10% oxygen and 4, respectively) an intermediate degree was apparent with less than 50% of the mice having nidatory sites. At 8% oxygen blastocyst implantation was observed. Uterine flushings of mice without implantation sites in Gps 3–5 contained cellular debris and occasionally blastocyst embryos. The flushed uterine horns of mice with implantation sites contained one to

three blastocysts in three of ten mice in Gp 3, two of ten mice in Gp 4, and four of nineteen mice in Gp 5. There was no difference in the number of embryonic swellings per uterus in any group when implantation sites were present at Day 8 of pregnancy.

Uterine and ovarian weights declined significantly as oxygen concentration was reduced (Table II). Mean CL weights in Gps 3 and 5 were significantly less than the 21% oxygen control. In Gp 5, only seven small CL could be dissected and weighed due to extensive luteal regression.

Responses symptomatic of hypoxia (adipsia, anorexia, hyperpnea, and polycythemia) were observed in Gps 2–5. Mean body weight of mice in Gp 1 increased by 6.3% during the exposure period, whereas Gp 2 mice maintained their body weight, and Gps 3–5 mice lost 10–20% of their initial weight (Table II). Between Days 6 and 8, mice in Gps 3–5 demonstrated a significant gain in body weight with the resumption of growth when compared to mice in the 21 and 14% oxygen groups. The mean hematocrit value of the groups at Day 6 of exposure was inversely related to the oxygen concentration to which mice were exposed, ranging from (mean \pm SE) 42.8 ± 0.52 at 21% oxygen to 59.1 ± 0.72 at 8% oxygen. The mean hematocrits at Day 8 in all groups were significantly less than the corresponding Day 6 means. The mean adrenal gland weight of the groups did not vary significantly.

Histologic observations. Hyperemic corpora lutea of pregnancy of Gp 1 mice were composed of irregular columns of homogeneous lutein cells. Individual lutein cells were composed of a large band of cytoplasm

TABLE 1. IMPLANTATION SITES AT DAY 8 OF GESTATION

Oxygen concentration ^a (%)	Total number of animals	Animals with implantation sites		Mean number of embryonic swellings per uterus of mice with implanted embryos \pm SE ^b
		<i>n</i>	% ^b	
21 (sea level)	68	48	70.5*	13.2 \pm 0.34*
14 (10,000 ft)	27	18	66.6* ^A	11.7 \pm 0.44*
12 (14,000 ft)	17	7	41.2 ^A	12.7 \pm 0.51*
10 (18,000 ft)	17	7	41.2 ^A	13.7 \pm 0.36*
8 (22,000 ft)	20	1	5.0 [†]	12.0 ^c

^a Approximate altitude in parentheses corresponding to the oxygen concentration.

^b Groups with different superscripts were significantly ($P < 0.05$) different.

^c Value is within the range of the means of Groups 1–4.

TABLE II. ORGAN AND BODY WEIGHTS

Group	Oxygen concentration ^a (%)	Uterine weight (mg \pm SE) ^{b,c}	Mean ovarian weight (pair) (mg \pm SE) ^{b,c}	Mean corpora lutea weight (μ g \pm SE) ^{b,c}	Body weight change between Days 0-6 (g \pm SE) ^{b,c}	Body weight change between Days 6-8 (g \pm SE) ^{b,c}
1	21 (sea level)	468.9 \pm 37.65 (21)*	17.8 \pm 1.18 (21)*	252.1 \pm 15.12 (76)*	+1.26 \pm 0.246 (26)*	+0.65 \pm 0.252 (26)*
2	14 (10,000 ft)	402.8 \pm 57.34 (11)*	13.7 \pm 1.17 (11)*	263.1 \pm 16.79 (32)*	+0.01 \pm 0.457 (10) ^a	+0.67 \pm 0.351 (10)*
3	12 (14,000 ft)	309.5 \pm 44.30 (16) ^a	11.2 \pm 0.65 (16) ^a	196.9 \pm 17.63 (35) ^a	-2.34 \pm 0.326 (16) [†]	+1.96 \pm 0.286 (16) ^a
4	10 (18,000 ft)	209.0 \pm 31.45 (17) ^a	14.7 \pm 0.74 (17) [†]	245.8 \pm 17.87 (38)*	-3.38 \pm 0.248 (17)*	+2.19 \pm 0.234 (17) ^a
5	8 (22,000 ft)	143.1 \pm 16.01 (19) [†]	8.5 \pm 0.59 (20)*	125.7 \pm 24.68 (7) ^a	-4.22 \pm 0.250 (20) ^{††}	+2.37 \pm 0.228 (20) ^a

^a Approximate altitude in parentheses corresponding to the oxygen concentration.^b Value in parentheses refers to number of observations.^c Group means with different superscripts were significantly ($P < 0.05$) different.

surrounding a vesicular nucleus. Corpora lutea of mice in Gps 2-4 were less hyperemic and had a smaller ratio of nuclear:cytoplasmic diameter when compared to Gp 1. In Gps 2-4 CL were incompletely luteinized, being composed of a heterogeneous cell population of vacuolated, pale-staining, lutein cells and small, intensely staining, eosinophilic cells. In Gp 5, lutein cells were highly vacuolated and the cytoplasmic:nuclear ratio was further reduced over Gp 1. These CL were just below the surface of the ovary, often infiltrated with connective tissue, and appeared to be retreating toward the medulla of the ovary.

Ovarian interstitial development was incomplete in mice exposed to oxygen concentrations of less than 12% when compared to Gp 1 controls. Small antral follicles were present in ovaries of mice in Gps 1-4. Numerous secondary and tertiary follicles undergoing atresia were present in the cortex of mice in Gp 5. The membrane granulosa of these follicles was only a few cell layers thick, or absent, and the antral cavity was frequently filled with blood.

In Gp 1, and usually in Gps 2-4, embryonic development proceeded to neurulation by Day 8. Embryonic resorption sites were more frequently observed in Gps 2-4. Pyknotic nuclei were present in embryonic and extraembryonic structures, and the uterine lumen contained an abundance of leucocytes. Uterine stromal hyperplasia and epithelial gland development were reduced in Gp 5 when compared to all other groups. In mice exposed to 8% oxygen an occasional free blastocyst was found in the cervix or adjacent to a crypt in the uterine horn.

Discussion. These experiments demonstrate that pregnancy may be terminated prior to implantation in the nonacclimated mouse exposed to oxygen concentrations of 12%. Embryonic implantation during hypoxic exposure appears to be an "all or none" response. Although the percentage of inseminated animals with implantation sites decreases as oxygen tension is reduced, those animals in which nidation occurs have a constant number of implantation sites regardless of the pO_2 treatment level. This constant number of implantation sites is governed by total ovulations and the normal

failure of a small number of blastocysts in rodents exposed to reduced oxygen during the *postimplantation* period. This response may range from the resorption or abortion of an individual fetus to the loss of an entire litter (2, 4). The number of offspring is dependent upon the severity of maternal hypoxia and the extent of acclimatization attained during exposure.

Changes in the size and morphology of the ovaries and the levels of LH and PRL in the CL indicate that secretion and/or release of luteotropic hormones [FSH and LH (9); LH and prolactin, (10)] to the ovary is depressed (Gps 2-4) or blocked (Gp 5). Follicular development is depressed when exposed to as little as 10% oxygen (equivalent to 18,000 ft) compared to control ovaries, indicating a decrease in FSH secretion. Thus, the reduction of prolactin (PRL) secretion may be a primary means by which luteal function is impaired. Regressing CL and numerous atretic follicles undergoing atresia in mice exposed to 8% oxygen may indicate that cyclic follicular development is depressed after conception, but ovulatory surges of LH were not available to the ovary. A similar selective inhibition of hypothalamic PRL and LH secretion has been demonstrated in the cycling hamster exposed to 10% oxygen at simulated altitude [oxygen concentration approximately 8.6%, (6)]. Depression of uterine weight during hypoxia is associated with nidation and reduced pregestational and embryonic support of pregnancy. A precise balance of progesterone in combination with estrogen is essential for sensitizing the endometrium for decidualization and implantation (11). The presence of free-floating embryos, reduced pregestational and regressing luteal tissue in mice exposed to 8% oxygen indicate that preparation of the uterus for implantation was inadequate. A nutritive environment incapable of maintaining viable embryos is implied because uterine flushings contained debris, leucocytes, and only a few embryos. In the nonpregnatorial uterus, most of preimplantation embryos are lost at the cervix on the day of ovulation of

subsequent cycles (12). In rare instances, blastocysts which appeared to be normal were observed in uterine crypts. Such embryos may be viable, since a 2-week delay of implantation in prepubertal mice (hormonally induced to ovulate and mate) may be followed by successful nidation upon progesterone administration (12).

Baird and Cook (4), using acclimated mice exposed to a simulated altitude of 14,000-25,000 ft, reported that resorption of fetuses was initiated when crown-rump length approached 7 mm [Day 12 of gestation (13)]. In the present study, exposure of the nonacclimated mouse to hypoxia resulted in the initiation of resorption before Day 8 of pregnancy when crown-rump length is less than 2 mm. This difference in susceptibility of the fetus in the nonacclimated mouse may be due to the altered trophic and endocrine support of gestation and to the direct effects of hypoxia on the conceptus prior to maternal acclimatization.

The progressively greater loss in body weight of pregnant mice produced by increasing severity of hypoxia may be due to several factors including reduced food intake, excessive loss of body water, and the direct effect of hypoxia on tissues. Recently, it was found that most of the reduction in weight of mice exposed to hypoxia was primarily loss of body water (14-16). It is difficult to establish unequivocally that hypoxia, per se, directly affects the ovary, uterus, or implantation. Severe nutritional deprivation has also been demonstrated to block implantation in the mouse (17). It has been shown that increased prenatal mortality occurs in rats exposed to 18,000 ft of simulated altitude (oxygen concentration approximately 10%) in the absence of any alteration in the normal gain in body weight (2). This suggests that loss in body weight during exposure to hypoxia (12 and 10% oxygen) may not be the primary cause of alterations in the reproductive system.

Summary. Inseminated mice were exposed to different degrees of hypoxia ranging from 8-14% during the first 6 days of gestation. The frequency of animals with embryonic implantation sites was reduced by 42% after exposure to either 12 or 10% oxygen and by 93% at 8% oxygen. When

implantation occurred, the number of nidatory sites per uterus did not differ significantly, which suggests an all or none response for blastocyst implantation. The failure of embryos to implant was associated with histologic evidence of luteal regression. An increased incidence of fetal resorption was apparent following implantation in mice exposed to 14, 12, or 10% oxygen. The reproductive dysfunction noted during hypoxic exposure is attributed to a substantial loss in body weight and to altered tropic and endocrine support of pregnancy.

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d Status and Carbonic Anhydrase Levels in Mouse Erythrocytes¹ (39497)RALPH H. STERN² AND RICHARD E. TASHIAN*of Biology, The Johns Hopkins University, Baltimore, Maryland 21218, and Department of Human The University of Michigan Medical School, 1137 E. Catherine Street, Ann Arbor, Michigan 48104*

alian carbonic anhydrase occurs in s, carbonic anhydrase I (CA I) and anhydrase II (CA II), which ap- e the products of two closely linked ici (1). The two isoenzymes differ ber of ways; in particular, the hy- f CO₂ is catalyzed more rapidly by in it is by an equivalent amount of . In human erythrocytes, carbonic e levels are influenced by the levels ine in the plasma. In hyperthyroid concentration of CA I is decreased ypothyroid individuals it is in- 3-9). To date, only a limited num- ports has appeared concerning the thyroid status on erythrocyte car- hydrase in experimental animals. In this report, we describe an ex- al model wherein alterations of tatus produce marked changes in te CA I concentrations of the use, *Mus musculus*.

Materials and Methods. Experimental ra- id animals. Our experiments in- e manipulation of thyroid status in id normal mice and the assessment s in erythrocyte CA I and CA II W/J is an inbred strain segregating cessive gene *dw*; homozygous *dw/dw* are panhypopituitary dwarfs. Hy- lism has been documented in this 3). To show that carbonic anhy- rations in *dw/dw* mice were due to thyroidism, some DW/J *dw/dw* re fed thyroid powder. Since (w/w, i.e., weight of supplement/ food) thyroid powder in a diet is to prevent goiter formation in low iodine diet (14), this quantity

should be the replacement dose for a severely hypothyroid mouse. It has previously been shown that DW/J *dw/dw* mice respond to thyroid hormone by an increase in weight (13), and this was also observed here.

C57BL/6J was chosen as a standard laboratory strain. A 0.1% (w/w) thyroid powder dietary supplement was arbitrarily chosen as a dose likely to produce hyperthyroidism. Propylthiouracil (0.1%) has been used previously in mice to produce hypothyroidism (14).

Two sets of experiments were performed, each with several groups of mice. In the first set, one group of six C57BL/6J females was kept on its regular diet (Jax 234 pellets, Old Guilford) while another group of six C57BL/6J females was fed the same diet supplemented with 0.1% thyroid powder (USP). For each group, the pellets were ground to a powder and mixed with the appropriate supplement in both sets of experiments. A final group of six C57BL/6J females was fed its regular diet supplemented with 0.1% (w/w) propylthiouracil.

In the second set of experiments, DW/J mice were used. Six DW/J ?/+ mice (three females and three males) and six DW/J *dw/dw* (three males and three females) were fed Jax 234 pellets, while seven DW/J *dw/dw* (three females and four males) were fed this diet supplemented with 0.066% thyroid powder (USP).

Feeding experiments were performed at the Jackson Laboratory, which is fully accredited by the American Association of Laboratory Animal Care. C57BL/6J mice were obtained from production colonies and DW/J mice were from the Mouse Mutant Stock Center. All mice were 2 to 3 months old at the start of the experiments. At the end of 46 days, the mice were exsanguinated.

Qualitative assessment of carbonic anhydrase. A sample of the blood from each

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animal was withdrawn and hemolysates were prepared from washed cells and examined by electrophoresis on Titan III cellulose acetate strips (Helena Laboratories) in a pH 9.1 buffer (25.2 g of Tris, 2.5 g of EDTA (acid) and 1.9 g of boric acid per liter) for 30 min at 300 V at room temperature. Protein was stained with Ponceau S. Since electrophoretic patterns from mice within a given treatment group appeared the same, blood from individuals of a group was pooled. Although no sex differences were visible, blood from males and females was pooled separately. Hemolysates were prepared and frozen at -80° for later quantitative measurement of each carbonic anhydrase.

Quantitative assessment of carbonic anhydrase. Concentrations of CA I and CA II were measured by radial immunodiffusion analysis, with the use of a modification of the technique described by Mancini (15). Antisera specific for mouse CA I and CA II were prepared by immunization of rabbits with purified enzymes. Mouse CA I and CA II were isolated from pooled hemolysates by chromatography on a CM-32 cellulose column equilibrated with 0.02 M phosphate buffer, pH 6.6. After addition of the hemolysate to the column, CA I and CA II were eluted with a linear 0 to 0.2 M NaCl gradient. Specific CA I and CA II antisera were prepared by injecting rabbits with the purified preparations of CA I and CA II as previously described (16). Specificity was established by showing the absence of crossreactivity between each carbonic anhydrase and antisera prepared against the other isozyme. An agarose solution was prepared by dissolving 0.225 g of agarose (MCI Biomedical, Rockland, Maine) in 14.625 ml of borate saline [prepared by mixing 1 vol of 0.125 M fused boric acid (B_2O_3), 0.075 M sodium chloride with approximately 0.5 vol of 0.125 M sodium borate ($Na_2B_4O_7 \cdot 10H_2O$), 0.075 M sodium chloride until the pH was 8.37] containing 0.1 mg/ml of thimerosal in a boiling water bath. After cooling the agarose solution to 50° , 0.375 ml of antisera was heated to 50° and quickly mixed with it. The resulting solution was quickly poured into the bottom (i.e., the wrong side) of an immunodiffu-

sion plate (Miles Laboratories, No. 42-150-1), uniformly distributed by tilting the plate, and allowed to cool to room temperature. The plate was allowed to sit overnight in a water-saturated atmosphere at room temperature. Excess fluid was then removed and wells of about 10- μ l vol were cut with a trochar (No. 10, Becton-Dickinson). The agarose plug was rimmed and removed with a 25-gauge syringe needle. Eight microliters of sample (standard or unknown) was placed in the wells. Radii of precipitin rings were measured after a 3-day incubation at room temperature in a water-saturated atmosphere. Measurement was facilitated by the use of a dissecting microscope fitted with an eyepiece reticle. A standard series of purified mouse CA dilutions was included in each plate. A regression line of precipitin ring area on carbonic anhydrase concentration of the standards was fitted to these data and used to calculate carbonic anhydrase concentration in the samples. This value was divided by the hemoglobin concentration of the hemolysates (determined by the absorption of cyanmethemoglobin at 545 nm) and the results were reported as μ g of CA/mg of hemoglobin. Multiple determinations were made for each experimental group.

Results. The results are shown in Table I. The experiments with C57BL/6J and DW/J mice were analyzed by one-way and two-way analysis of variance, respectively. The only significant results were treatment effects for CA I. Erythrocyte CA I concentrations in control C57BL/6J mice were significantly less than those in C57BL/6J mice fed propylthiouracil ($P < 10^{-4}$) and significantly greater than those in C57BL/6J mice fed thyroid powder ($P < 10^{-2}$). Erythrocyte CA I concentrations in untreated DW/J *dw/dw* mice were significantly greater than those in DW/J *+/?* mice ($P < 10^{-7}$) and those in DW/J *dw/dw* mice fed thyroid powder ($P < 10^{-8}$). These multiple comparisons are not statistically independent (i.e., orthogonal).

Discussion. The data show that hypothyroidism, whether produced genetically or pharmacologically, elevates CA I levels. Moreover, feeding genetically hypothyroid mice thyroid powder lowered their elevated CA I levels. Similarly, thyroxine injections

TABLE I. EFFECT OF THYROID POWDER AND PROPYLTHIOURACIL (PTU) ON ERYTHROCYTE CARBONIC ANHYDRASE ISOZYMES, CA I AND CA II, IN DIFFERENT MOUSE STRAINS.

Strain	Genotype ^a	Supplement	Sex	CA I ^b (μg/mg Hb)	CA II ^b (μg/mg Hb)
C57BL/6J	+/+	None	Female	3.7 (3.4-4.0)	7.1 (6.2-7.8)
C57BL/6J	+/+	PTU ^c	Female	7.2 (6.0-8.4)	7.1 (6.8-7.3)
C57BL/6J	+/+	Thyroid	Female	1.9 (1.6-2.1)	7.4 (6.7-8.3)
DW/J	+/?	None	Male	3.5 (3.2-3.9)	6.1 (5.8-6.4)
DW/J	+/?	None	Female	2.7 (2.4-3.1)	6.2 (5.9-6.4)
DW/J	<i>dw/dw</i>	None	Male	7.8 (6.2-8.8)	5.9 (5.8-6.0)
DW/J	<i>dw/dw</i>	None	Female	7.9 (6.3-9.1)	5.7 (5.6-5.9)
DW/J	<i>dw/dw</i>	Thyroid	Male	2.1 (2.0-2.1)	5.7 (5.3-5.9)
DW/J	<i>dw/dw</i>	Thyroid	Female	1.7 (1.6-1.8)	6.0 (6.0-6.1)

^a Refers to genotype at *dw* locus.

^b Values given are means of three or four determinations. Values in parentheses refer to the range of determinations on the pooled samples.

^c Propylthiouracil.

have been reported to decrease erythrocyte CA I levels in the rabbit (10). On the other hand, the injection of thyroxine for up to 34 weeks into pig-tailed macaques (*Macaca nemestrina*) failed to produce any reduction in red cell carbonic anhydrase levels (12). In experiments where the synthesis of CA I and CA II was followed in reticulocytes of the rhesus macaque (*Macaca mulatta*) and *M. nemestrina*, no alteration in the translation of CA II or two allelic forms of CA I as observed on addition of thyroxine to the reticulocyte systems (12, 16). It was of interest, however, that the synthesis of one genetic variant of CA I in *M. nemestrina* was reduced with thyroxine treatment (2). In the present study, mouse red cell CA II levels did not appear to be altered by any of the test procedures, a finding which seems to be in contrast to an apparent positive correlation between the altered levels of human red cell CA I and CA II in hyper- and hypothyroid states (3, 6, 7, 10). This difference between the effect of thyroxine on CA II in mice and humans may be due to differences in their regulatory control mechanisms.

The effects described here are probably maximal since studies in man indicate that alterations in erythrocyte carbonic anhydrase concentrations occur during formation and/or maturation of the erythrocyte (1). By analogy, maximal results should appear after 60 days, the lifetime of a mouse erythrocyte, slightly later than the 46 days treatment used here. Furthermore, different dietary protocols might produce larger alterations in erythrocyte CA I.

Summary. Carbonic anhydrase I (CA I) levels in mouse erythrocytes are influenced by thyroid status, while carbonic anhydrase II (CA II) levels appear to be unaffected. Adding propylthiouracil to the diets of normal mice (C57BL/6J) resulted in elevated erythrocyte CA I levels, while thyroid powder produced decreased levels. Panhypopituitary mutants (DW/J *dw/dw*) have increased levels of erythrocyte CA I in comparison to their normal litter mates (DW/J +/?). These increased levels were decreased by adding thyroid powder to their diets.

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Comparison of γ -Butyrolactone and Pimozide on Serum Gonadotrophins and Ovulation in the Rat (39498)

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lytic cleavage of γ -butyrolactone *in vivo* produces the unique CNS agent, γ -hydroxybutyrate (GHB) (1). Both compounds produce CNS depression, this latter compound is exceptional in that it is a normal brain metabolite and produces a significant increase in dopamine (DA) without affecting other brain neurotransmitters such as norepinephrine (NE), serotonin (5HT), or γ -aminobutyric acid (GABA) (3-6).

Of the postulated role the dopaminergic-infundibular system plays in gonadotrophin release and subsequent ovulation (10), we have compared the effects of γ -butyrolactone and pimozide, a fairly specific dopamine receptor blocker, on the preovulatory gonadotrophin surge and ovulation. GBL passes the blood-brain barrier and is believed to be the active moiety (1, 11). We have employed GBL since it is immediately hydrolyzed to GHB in plasma and is more rapidly and uniformly distributed than GHB after intraperitoneal injection (1). Although high doses of pimozide previously have been reported to delay ovulation and reduce the preovulatory LH surge (13), no dose relationship has been made between an anti-ovulatory effect and the preovulatory gonadotrophin surge. **Materials and methods.** Mature female Sprague-Dawley (S-D) rats (Charles River, Woburn, Massachusetts), 225-250 g in weight and acclimated to laboratory conditions were maintained on a fixed 14-hr light/dark lighting schedule (lights off at 1330 hr). Only those rats exhibiting at least five consecutive 4-day cycles were used. γ -Butyrolactone (n_D^{20} 1.4365, Aldrich Chemical Co.) was diluted with saline and injected intraperitoneally at 1330 hr on proestrus. Pimozide (1-[4,4-bis(*p*-fluorophenyl)butyl]-2-benzimidazolinone; Janssen Pharmaceutica) in corn oil was injected subcutaneously at 1330 hr on proestrus. Sequential blood sam-

ples for determination of serum LH and FSH by RIA (14) were taken by substernal heart puncture (0.5-1.0 ml; volume replaced by saline) under light ether anesthesia hourly from 1330-1730 hr proestrus. In our hands, cardiac puncture, via substernal entry, does not overcome pentobarbital's blockade of ovulation (15), nor is there a significant difference in serum LH and FSH between rats with indwelling cannulae (16) and cardiac puncture (C. W. Beattie, unpublished observations). All values for serum LH and FSH are reported irrespective of whether the animal ovulated. Autopsies were performed on the morning of expected estrus and the degree of ovulation was assessed by counting tubal ova.

Serum LH and FSH levels were determined by RIA from respective kits supplied by the NIAMDD Rat Pituitary Hormone Program and by Dr. A. Parlow. LH was assayed from duplicate 25- μ l samples. FSH was determined in duplicate 50- μ l samples. The lower limit of sensitivity for both hormones was 10 ng/ml. Values were expressed in terms of LH and FSH RP-1, respectively.

Results. The degree of ovulatory inhibition produced by increasing doses of GBL is illustrated in Table I. The antiovarian ED_{50} was approximately 250 mg/kg, which is a subanesthetic dose. At this dose, increases in uterine wet weight (luminal fluid expressed) accompanied the increased incidence of uterine ballooning, but only the 750-mg/kg dose of GBL produced a significant increase over control. No change was noted in ovarian weight.

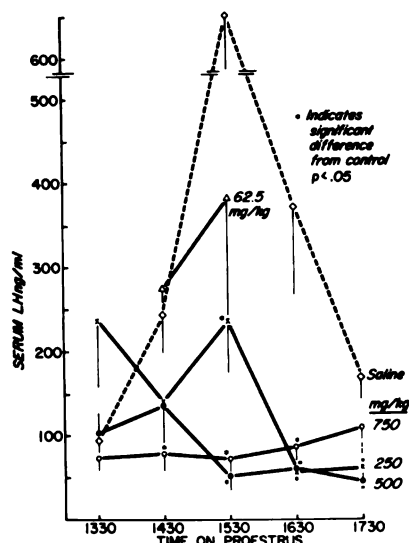
Proestrous serum LH levels from the rats described in Table I are illustrated in Fig. 1. GBL produced a significant dose-related decrease in serum LH levels over the time period sampled. By 1630 hr, proestrous FSH levels (Fig. 2) were significantly reduced by doses of GBL above the antiovarian ED_{50} .

TABLE 1. INHIBITION OF OVULATION WITH γ -BUTYROLACTONE (GBL) OR PIMOZIDE.

Drug	mg/kg	N	Number of rats ovulating	Percentage inhibition of ovulation	Number of ova in ovulating rats
Saline	—	15	15	0	13.2 \pm 0.5 ⁺
GBL (ip)	750*	5	0	100	0
	500*	7	2	71	13, 15
	250	11	4	63	11.7 \pm 0.9
	125	5	4	20	9.5 \pm 1.6
	62.5	9	7	22	12.3 \pm 0.7
Oil (sc)	—	5	5	0	12.6 \pm 0.9
Pimozide (sc)	2.5	6	0	100	0
	1.25	6	2	67	11, 12
	0.75	5	3	40	7, 13, 8
	0.63	4	4	0	10.0 \pm 1.0

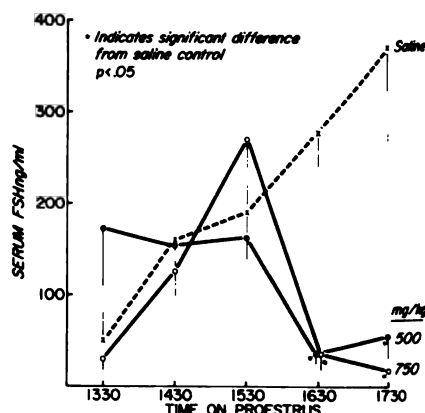
⁺ Mean \pm standard error.

* Anesthetic dose.

FIG. 1. The effects of γ -butyrolactone (ip) on the proestrous serum LH surge. Each point represents five to eleven animals. Values are expressed as mean \pm SE.

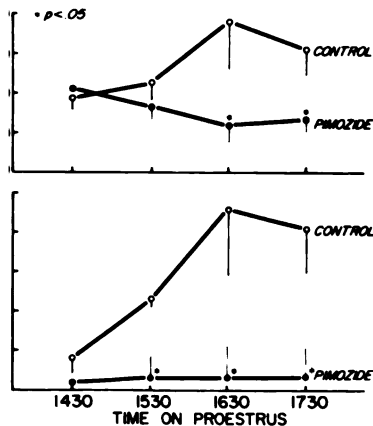
Pimozide significantly reduced proestrous serum FSH and LH values (Fig. 3) over the sampling period at its approximate antiovaratory ED₅₀ doses (Table I).

Discussion. These data demonstrate for the first time that GBL blocks ovulation. Since GHB appears to be the active moiety (1, 11, 12), it is likely to be responsible for the suppression of the proestrous gonadotrophin surge and subsequent inhibition of ovulation. While the exact mechanisms and

FIG. 2. The effect of γ -butyrolactone on the proestrous serum FSH surge. Each point represents five to eleven animals. Values are expressed as mean \pm SE.

sites of the GBL blockade of ovulation are unknown, it appears that GBL, like pimozide, inhibits ovulation by suppressing the CNS-induced proestrous gonadotrophin surge.

The neuropharmacology of GBL differs markedly from pimozide. GBL or GHB produces an increase in brain DA content which is accompanied by a reduction in impulse flow within central dopaminergic neurons (17), coupled with a decrease in DA release and/or metabolism (6). In this respect, it differs from pimozide, which selectively decreases brain DA concentration at low doses (18). Clearly, an alteration in brain DA content per se is not directly re-



The effect of pimoziide (1.25 mg/kg sc) on LH and FSH levels of proestrous rats. Each sample is from four to six animals. Values are mean \pm SE.

ovulation inhibition or to a decrease in the oestrous surge of gonadotrophins. If one considers only the turnover of central dopamine, one finds that GHB appears to decrease the turnover of DA (5, 6), while GBL increases DA turnover (18). It is therefore, that neither DA content nor its turnover per se affect the gonadotrophin release and subsequent ovulation. The single thread of control administration of either of these affecting dopaminergic impulse flow at any rate appears to be that any interference, pre- or postsynaptically, of central dopaminergic nerve impulse flow over a discrete period, alters gonadotrophin release and subsequent ovulation.

It is noteworthy that pimoziide only began to decrease proestrous serum gonadotrophin levels and inhibit ovulation at doses comparable upon central NE content and turnover (18). Barbiturates also begin to interfere with (cortical) NE function (19) at similar doses (20).

Collectively, the present studies suggest that in the intact proestrous rat any interference of central dopaminergic impulse flow significantly decreases LH and FSH levels and inhibits ovulation. These results suggest that in the intact animal, effects of putative dopaminergic blockers affect noradrenergic as well as

dopaminergic neurons, are necessary to significantly reduce the proestrous gonadotrophin surge and subsequently ovulation.

Summary. Proestrous serum LH and FSH levels and ovulation were significantly reduced when either γ -butyrolactone (GBL), which is hydrolyzed *in vivo* to the naturally occurring CNS depressant γ -hydroxybutyrate (GBH), or pimoziide, a dopaminergic receptor blocker, was administered to 4-day cyclic rats just prior to the proestrous critical period. These data suggest that GBL and pimoziide inhibit ovulation by decreasing the proestrous serum LH and FSH surges through effects on a central dopaminergic pathway.

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Plaque Assay for *Rickettsia mooseri* in Tissue Samples¹ (39499)

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me-honored methods of animal or
ated egg inoculation for quantitat-
nfectivity of rickettsial suspensions
bersome and time consuming and
hibited the study of basic mecha-
rickettsial diseases. However, the
nent of a plaque assay for rickett-
McDade (4) on the basis of Kor-
riginal brief report (3) and its re-
by Wike *et al.* (9, 10) have pro-
sensitive and relatively simple pro-
the isolation and quantitation of
ents. The successful application of
techniques for the quantitative isola-
Rickettsia rickettsii from tick hemo-
id from guinea pig whole blood (8)
this procedure to be useful when
to study of rickettsial infections in

This paper describes the applica-
the plaque technique to the direct
of *Rickettsia mooseri* (*R. typhi*)
ole blood and other tissues of in-
guinea pigs.

Materials and methods. Rickettsiae. A
of *R. mooseri*, Wilmington strain,
pared by homogenizing infected
s in sucrose phosphate glutamate
) solution to produce a 50% (w/v)
suspension. The homogenate was
d into glass ampoules which were
aled, quick-frozen in a dry ice-alco-
ure, and stored at -70° until used.
ettsiae employed for these studies,
from Dr. Richard A. Ormsbee of
y Mountain Laboratory of the Na-
stitutes of Health, had a passage

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history of 12 embryonated egg, 15 guinea
pig, and 5 embryonated egg passages
(12EP/15GP/5EP). A partial characteriza-
tion of this seed is presented in Table I.

Quantitation of Rickettsiae. 1. *Rickettsial particle counts.* A modification (Fiset *et al.*, unpublished) of the method of Silberman and Fiset (6) was used to count rickettsiae in crude yolk sac suspensions.

2. *Guinea pig intradermal ID₅₀ titrations.* Male Hartley guinea pigs of approximately 500 g (obtained from R. C. Roscrans, Hamilton, Montana) were used for all titrations. The stock egg seed was diluted with SPG and 0.1-ml intradermal (id) inoculations were employed for infection of guinea pigs. Tissue homogenates, diluted with 3.7% brain heart infusion (BHI) (Baltimore Biological Laboratories), were inoculated id in 0.2-ml amounts. Animals were bled before, and 28 days after, inoculation. Infection was determined by measuring serologic conversion using the microagglutination (MA) test (see below). All preinoculation samples displayed MA titers of $\leq 1:2$. A titer of 1:4 or higher in the 28-day serum was considered positive and indicative of infection. The ID₅₀ on the basis of serologic conversion was calculated by the method of Reed and Muench (5) and is expressed as 50% guinea pig intradermal infectious doses (GPI-DID₅₀).

3. *Plaque assay technique.* The primary chicken embryo (CE) fibroblast plaque assay technique employed was a modification of the procedures described by McDade (4) and Wike *et al.* (9, 10). Half-strength Dulbecco's modification of Eagle's minimal essential medium with Earle's salts (7) containing 0.1% glucose and 5% fetal calf serum was substituted for the M-199 medium and 60-mm plastic petri dishes (Falcon Plastics) were used instead of flasks. The petri dishes were incubated for 12 days at 32° in moist air containing 5% CO₂. No antibiotics were used. Tissue samples to be titrated by

plaque assay were used undiluted (10% w/v suspensions in BHI, see below) and diluted 1:5, 1:10, 1:100, 1:1000 and 1:10,000 with BHI. Each dilution was plated in five petri dishes, 0.2 ml per dish. One hour, at room temperature with rocking every 15 min, was allowed for adsorption.

Serologic techniques. The microagglutination test (2) was used with ether-treated, "specific" particulate *R. mooseri* antigens at a concentration of 333 $\mu\text{g/ml}$. The diluent for antigen and for serum dilutions was 0.9% NaCl which contained 0.01% merthiolate and 1% nonimmune guinea pig serum.

Preparation of tissues. Whole blood and solid tissues were harvested aseptically. Solid tissues were washed free of loosely adherent materials and blood with chilled sterile physiological saline and homogenized in BHI to produce 10% (w/v) suspensions. With the exception of skin biopsies, homogenization was performed in an Omni-mixer (Sorvall). The Omni-mixer canister was immersed in a water-ice mixture and the unit was operated at maximal speed for two 1.5-min cycles separated by a 1.5-min cooling period. When skin was used for plaque assay, the area of interest was shaved, the animal was dipped in water containing 5% disinfectant (OsyI, National Laboratories), dried, treated with a depilatory agent (Nair, Carter Products), painted with 2% tincture

of iodine solution, and rinsed with sterile water. Skin biopsies were obtained using an 8-mm circular cutaneous punch, the depth of the biopsy being determined by the cleavage plane between the subcutaneous fat and the subcutaneous musculature. Biopsies obtained in this manner were thoroughly disrupted in a Ten Broeck tissue grinder.

Suspensions which were not assayed immediately were shell frozen in rubber-stoppered vaccine bottles in a dry ice-alcohol mixture and were stored at -70° .

Results. Recovery of *Rickettsiae* from tissues of infected guinea pigs. Animals were sacrificed prior to infection and 3 and 10 days after intraperitoneal inoculation with 2.55×10^6 PFU of the *R. mooseri* seed. The tissue homogenates from these animals were assayed in the CE cell system. Homogenates from animals that had not been infected did not produce plaques in CE cell monolayers. However, after infection, plaques were readily obtained from selected tissues and the number of plaques varied with both tissue and time after infection (Table II). In addition to the results recorded in Table II, we have also been able to count rickettsial plaques from the lymph nodes, kidney, and skin following intradermal infection.

Comparison of plaque assay and GPI-DID₅₀. The sensitivity of the plaque assay for quantitating rickettsiae in tissues was compared to the GPI-DID₅₀ as determined by serologic conversion. This was accomplished by simultaneous titration of samples in both CE cell cultures and in guinea pigs. The PFU was found to be a more sensitive measure of viable rickettsiae than the GPI-DID₅₀ (Table III). As Table III shows, the titer by plaque assay was in all cases higher

TABLE I. CHARACTERIZATION OF 12EP/15GP/5EP *R. mooseri* SEED

Character	Number per milliliter
Rickettsial particles	1.23×10^{10}
Plaque-forming units	2.55×10^8
Guinea pig ID ₅₀	2.65×10^8

TABLE II. DETERMINATION OF RICKETTSIAL PLAQUE-FORMING UNITS IN VARIOUS GUINEA PIG TISSUES AFTER INTRAPERITONEAL INFECTION WITH 2.55×10^6 PFU OF *R. mooseri*

Tissue	PFU ^a on day after infection (days)		
	0	3	10
Spleen	$<2.50 \times 10^{2b}$	1.72×10^4	6.88×10^2
Myocardium	$<2.50 \times 10^2$	1.11×10^3	4.63×10^2
Fat pad	$<2.50 \times 10^2$	2.32×10^3	7.50×10^2
Whole blood	$<2.50 \times 10^1$	3.75×10^2	8.75×10^2

^a PFU per gram of tissue (wet weight) or ml of blood.

^b The minimal number of PFU per gram of tissue was 2.50×10^2 and the minimal number of PFU per ml of blood was 2.50×10^1 because of the dilution factor and the imposition of a limit of 25 PFU/ml as the minimal number which was considered significant.

TABLE III. SENSITIVITY OF PLAQUE ASSAY VERSUS GUINEA PIG INFECTIOUS DOSE FIFTY

Sample	Titer ^a		Ratio of PFU/ID ₅₀
	PFU	ID ₅₀	
Testicular fat pad	3.68×10^4	3.22×10^3	11.43
Spleen	6.88×10^2	3.22×10^1	21.37
Skin	2.68×10^2	1.65×10^2	1.62
Kidney	5.75×10^2	5.00×10^2	1.15

Values are expressed as the number of units per milliliter of undiluted tissue homogenate (10%, w/v, in 3.7%

TABLE IV. EXAMPLES OF INHIBITION OF PLAQUE FORMATION

Tissue	Dilution of sample	Number of PFU per Petri dish	PFU per gram of tissue or ml blood	Plaque recovery (percentage of maximum)
Skin biopsy ^a	Undiluted ^a	69.3	3,465	38.5
	1:5	25.0	6,250	69.4
	1:10	18.0	9,000	100.0
Kidney ^a	Undiluted	19.6	980	4.3
	1:5	69.6	17,400	85.7
	1:10	40.6	20,300	100.0
Whole blood	Undiluted	5.2	260	1.3
	1:10	40.3	20,000	100.0
Spleen ^a	Undiluted	80.0	4,000	13.2
	1:10	60.5	30,250	100.0

diluted samples are 10% (w/v) homogenates of the respective tissues in 3.7% BHI.

The titer arrived at through GPIDID₅₀ assays. The relative sensitivity of the assay to GPIDID₅₀ varied with the tissue and ranged from 21 PFU/GPIDID₅₀ in spleen to 1.2 PFU/GPIDID₅₀ in skin, when skin homogenates were used for titration.

Inhibition of plaque formation. In the course of titration of certain tissue homogenates and in addition to the variation in GPIDID₅₀ with tissue noted above, a discrepancy was noted between the numbers of PFU recovered from concentrated and diluted samples. This is illustrated in Table I where it is shown that some undiluted tissue suspensions display fewer PFU per milliliter than the corresponding 1:10 dilutions. The undiluted tissue suspensions seem to exert an inhibitory effect.

Bacterial and fungal contamination. Because antibiotics are not included in the medium employed for rickettsial plaque assay, contamination by bacteria and fungi is a potential problem. In addition to the contaminants randomly introduced during the plating procedure, certain tissues present particular problems. Skin, for exam-

ple, routinely showed bacterial contamination in low dilutions. Spleen and kidney homogenates, on the other hand, sporadically yielded a few fungal colonies. The contamination of skin homogenates with bacteria could at times interfere with the quantitation of rickettsiae, when low numbers of rickettsiae were present. With strict attention to aseptic technique, however, bacterial and fungal contamination was a relatively small problem in the more dilute samples.

Discussion. This study has shown that it is possible to obtain *R. mooseri* plaques directly from infected guinea pig tissue homogenates, that the number of plaques varies from tissue to tissue at a given time after infection, and that the number of plaques varies in a given tissue at different times after infection. These results show that the chicken embryo fibroblast plaque assay method is capable of detecting rickettsiae and enumerating the relative number of rickettsiae in guinea pig tissues. Because the plaquing efficiency of rickettsiae is less than 1 and because multiple factors may contribute to this inefficiency, the reliability of the method requires scrutiny.

A simple method has been devised for determining the absolute rickettsial body count of purified suspensions (2). However, no reliable method has yet been devised to quantitate the absolute concentration of viable rickettsiae. Instead, it has been necessary to quantitate viability in terms of infectivity for animals or cells, and infectivity values vary with the system employed. For example, the ratio between PFU titers in CE cells and the 50% guinea pig infectious titers as determined by serological conversion appears to vary with the source of the inoculum. Simultaneous titrations of *R. mooseri* from stock yolk sac seeds have shown the guinea pig infectivity titrations measured by serologic conversion to be as sensitive (see Table I) or more (10) sensitive than plaque titrations. However, the plaque titration is the more sensitive measure of rickettsiae in homogenates of guinea pig tissues (see Table III). In addition to this variability between systems, we found that recovery of rickettsiae from some tissues depends upon the dilution of the tissue homogenate. This suggests that tissue homogenates may contain a factor(s) that differentially inhibits plaque-forming capacity.

The presence of antirickettsial antibodies in the tissue homogenates could, possibly, be responsible for the guinea pig ID₅₀ being less sensitive than the plaque titration when rickettsiae in tissue homogenates are being titrated. Antibody has been shown to be capable of reducing infectivity for mice (C. L. Wisseman, Jr., D. W. Krause, I. B. Fabrikant, and P. A. Mackowiak, manuscript) but does not inhibit plaque formation in the chicken embryo fibroblast plaque assay (12). Two observations suggest that the inhibitor of plaque formation observed in these experiments is not antibody: (1) The inhibition is lost upon dilution of the tissue suspension containing the rickettsiae; and (2) competition by host cell fragments, presumably membranes, has been shown by others in these laboratories to be capable of reducing plaque titers (12). The high (10%) tissue content of the homogenates suggests the presence of a high concentration of host cell membrane fragments. The influence of host cell membrane concentration on guinea pig infectivity is unknown. Other undiscovered

inhibitory factors may also be present in crude animal tissue homogenates.

Because the number of plaques recovered from a given tissue varies with dilution, it is necessary to prepare the tissue homogenates in a standardized fashion. Others have demonstrated (11) that the removal of host cell membranes from suspensions containing rickettsiae removes the membrane-associated inhibitory effect. Indeed, in some preliminary experiments in the present study (data not shown), mixing 10% normal guinea pig kidney homogenate with a rickettsial suspension reduced the number of recoverable plaques. Low speed centrifugation, which undoubtedly contained host cell membrane fragments, retained some inhibitory activity. This suggests that the development of techniques to remove guinea pig cell components selectively from the suspensions containing the rickettsiae would result in an increase in the efficiency of the plaque assay for rickettsiae from these tissues.

The observed increase in recovered PFU with dilution of tissue homogenate suggests that the effect of inhibitory factors in the homogenates decreases with reduction in concentration more rapidly than the capacity of rickettsiae to form plaques. From a practical point of view, therefore, the highest dilution of a standardized tissue homogenate that yields plaques should be chosen to calculate the relative plaque titer of the tissue.

The variation between tissues as to capacity for inhibition of plaque formation (see Table III) makes comparison of relative number of PFU in one organ as compared to another of questionable validity. However, under standardized conditions, the comparison of the number of PFU recovered from an organ of one animal as compared to the number of PFU recovered from the same organ of similarly infected animals has been shown to be a highly reproducible value (J. R. Murphy, C. L. Wisseman, Jr. and P. Fiset, in preparation).

Summary. A plaque assay using primary CE fibroblasts was shown to be sensitive for the direct isolation and quantitation of the relative number of *R. mooseri* from infected guinea pig tissues.

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Effect of Local Administration of Levan on Skin Homograft Rejection in Mice (39500)

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Various immunosuppressors have been used to prolong the survival of homografts and thereby delay the rejection of implanted organs and tissues. Levan, a high molecular weight polysaccharide, has been shown to inhibit the inflammatory response by preventing both transport of proteins, including immunoglobulins (1, 2), and diapedesis of cells across the endothelium (3). Repeated intraperitoneal injections of levan were found to delay homologous skin graft rejection in mice significantly (4). The present work describes the effects of local subcutaneous injections of levan on graft rejection.

Materials and methods. Balb/C male mice, 8-10 weeks old, were used as recipients and as donors of isologous skin. Homologous skin samples were taken from C3H mice.

Each animal was grafted with isologous skin on one side of the back and homologous skin on the other side. The isografts served as controls for the skin-grafting technique.

Skin transplantation was performed according to Billingham and Silvers (5), with the addition that residual hair was removed by BaS and the skin was cleansed with ethanol and ether after shaving. Grafts were obtained with a punch, 5 mm in outer diameter. Micropore surgical tape (Blenderm 3M Company, St. Paul, Minnesota) was placed over the transplant with a cellophane window to permit daily inspection. The cellophane was prevented from adhering to the graft by a plastic ring (10 mm across and 2 mm thick) placed between them.

Native levan was obtained from the Technical Unit, Department of Biological Chemistry of the Hebrew University in Jerusalem. Five percent levan solution in saline was

prepared according to Shilo, Wolman, and Wolman (3).

Levan was administered either intraperitoneally (ip) or by subcutaneous (sc) injection:

One group of mice received ip injections of 10 mg of levan on Days 8, 6, and 4 before grafting and 25-mg daily sc injections beginning 1 day before grafting until homograft rejection.

Other groups of mice received daily sc injections of 5 or 10 mg close to (<1.5 cm) or far from (>4 cm) the graft site, beginning 1 day before grafting and continuing until rejection.

Results. The effects of ip and local sc injections of levan are summarized in Table I. The mean survival time (MST) of the grafts in the control untreated mice was 10.1 days. Daily sc injections of 5 mg of levan adjacent to the skin graft increased MST to 15.5 days, and 10 mg increased it to 17.2 days. A dose of 10 mg injected at a distance from the skin graft, in which any possibility of local damage to the graft was eliminated, yielded an MST of 17.8 days. When given ip, a dosage of 25 mg of levan was required to obtain a similar effect (MST of 16.6 days).

The distribution of survival times of animals treated by various routes and dosages of levan is illustrated in Fig. 1. In the untreated control mice, 87% of the homografts were rejected by Day 11, while none of the grafts in the levan-treated animals was rejected at that time. The rejection delay was dose dependent, as can be seen in animals given different amounts of levan at the same distance from the graft. One hundred percent graft rejection occurred on Day 17 with a 5-mg daily dose of levan, while with 10 mg this occurred on Day 22. No significant differences were noted between sc injections adjacent to or remote

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TABLE I. THE EFFECT OF VARIOUS ROUTES OF LEVAN ADMINISTRATION ON THE MEAN SURVIVAL TIME OF SKIN HOMOGRAFTS

Group	Dosage (mg)	Route of administration	Number of mice	Mean survival time (MST in days)	P
Control	—	—	15	10.1 ± 1.4	
n	25	ip	11	16.6 ± 2.7	<0.005
n	5	sc ^a	12	15.55 ± 1.1	<0.005
n	10	sc ^a	11	17.2 ± 2.9	<0.005
n	10	sc ^b	19	17.8 ± 2.0	<0.005

^a 4 cm from the graft.
^b 1 cm from the graft.

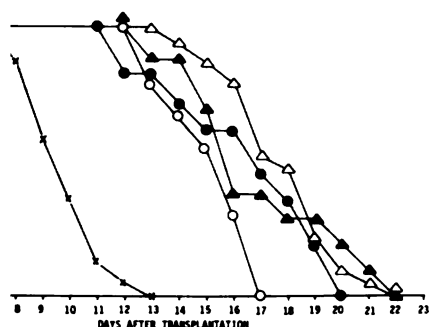


Fig. 1. Effect of levan on survival of skin grafts in mice: x, control; ●, 25 mg ip; ○, 5 mg local; ▲, local; △, 10 mg >4 cm from the graft.

the transplant. Intraperitoneal injection of 25 mg delayed 100% rejection than local injections of 5 mg, but less than 10-mg injections (20, 17, and 22 days respectively).

Discussion. The inhibitory effect of levan administered ip on graft rejection has been reported in a previous work (4). In the possible damage that ip injection cause to the abdominal organs, the intraperitoneal administration was considered as an alternative.

Intraperitoneal administration of a dose as low as 10 mg had an inhibitory effect on rejection similar to 25 mg of levan administered ip ($0.4 > P > 0.3$). Even 5 mg of sc levan inhibited the graft rejection to nearly the same extent ($0.1 > P > 0.05$). These results indicate that local sc injections of levan are at least as effective as intraperitoneal administration. In other experiments in our laboratory (unpublished results) the level of levan in the blood was found to be much lower when injected sc than when injected ip with the presently used doses and routes. This indicates that the inhibitory

effect of levan on graft rejection primarily depends on the local concentration of levan in the graft bed area.

In view of the possibility that repeated injections of levan administered to the neighborhood of the graft bed might mechanically affect graft survival, we also injected levan sc at a distance from the grafted area. In this experiment the delay in rejection time was similar to that of mice injected with levan near the graft ($0.3 > P > 0.2$). We concluded that local sc injection of levan causes no physical damage to the graft.

Summary. The influence of subcutaneous levan administration on the survival time of homografts was compared to that of intraperitoneal administration of levan. It was found that the inhibitory effects on rejection produced by a high dosage of levan administered intraperitoneally were similar to those produced by a much lower subcutaneous dose. Similar inhibitory effects were observed when levan was injected subcutaneously close to or far from the graft, and it was concluded that the inhibition was not a result of physical damage to the graft bed or mechanical interference with the rejection process.

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Serum Glycerol and Hepatic Glycerokinase Activity in the Carbohydrate-Sensitive BHE Strain of Rat¹ (39501)

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The carbohydrate-sensitive BHE² strain of rat is presently being used by this laboratory as a model of the interrelationship between carbohydrate and lipid metabolism. Rats of this strain have been shown to develop hyperlipemia and to exhibit increases in synthesis and storage of carcass and liver lipid when fed a high carbohydrate diet (1). BHE rats also respond to different kinds and amounts of dietary carbohydrate with substantial increases in hepatic lipogenic enzyme activity before the actual onset of hyperlipemia. Recent studies indicate that the nonfasted rats of this strain also exhibit elevated serum free glycerol levels (2). Similar elevations in serum free glycerol have also been observed in Zucker "fatty" rats during *in vitro* fat mobilization studies (3). These increases in glycerol in the Zucker rats have been attributed to excessive adipose tissue lipolysis. This does not seem to be the case with the BHE strain, however, since data recorded for this strain suggest that the metabolic error resides in the liver rather than the adipose tissue (1, 4).

Uptake of glycerol by the tissues is regulated by several factors including the concentration of glycerol in the blood (5), the nutritional state of the animal (6), and the simultaneous metabolism of other substrates (7). Glycerokinase (EC 2.1.1.30), which is found primarily in the liver and

kidney, is involved in the phosphorylation of glycerol to α -glycerophosphate by Glycerokinase is thus directly involved in the regulation of glycerol uptake and utilization by these tissues (8).

This study was undertaken to determine whether the elevated serum free glycerol found in the nonfasted BHE rats was related to hepatic glycerokinase activity.

Materials and methods. For each of the experiments, 45-day-old male BHE Wistar (Grand Island Biological Laboratory, Madison, Wisconsin) rats were housed individually in wire mesh cages in a temperature-humidity controlled room. Light was regulated so as to provide equal periods of light and dark. Animals were fed a laboratory chow diet (Purina laboratory rat chow, Ralston Purina Co., St. Louis, Missouri) *ad libitum*. At 75 days of age the animals in the nonfasted state were sacrificed after anesthesia with 60 mg of sodium amobarbital. Body weight, the thoracic cavity was opened, and blood was drawn by puncture. The sera collected after centrifugation (4°, 15 min, 3000 g) were used for the determination of free glycerol (9). Livers were quickly removed, placed in preweighed bags, fast-frozen in methanol-dry ice, and stored at -80° for not more than 3 days. Sections of the fast-frozen liver tissue were homogenized in 2 vol of cold (w/v) KCl-EDTA. Glycerokinase activity was assayed by the radiochemical method of Newsholme *et al.* (10), which is based on the conversion of ¹⁴C glycerol (glycerol-¹⁴C-3, 30 mCi/mmol, Amersham Pharmacia Corporation, Arlington Heights, Ill.) into L-[3-¹⁴C]glycerophosphate and the detection of the latter on DEAE cellulose disks. Supernatants prepared from liver homogenates by ultracentrifugation (100,000 g) were used as the source of glycerol in the first experiment. For the second experiment, glycerokinase activity was

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² The BHE is a strain of rat resulting from a cross between the Pennsylvania State College strain and the Osborne-Mendel (also called Yale) strain. These animals are currently available from Floco Laboratories, Dublin, Virginia. (Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.)

mined from crude liver homogenates without further treatment (10) L- α -glycerophosphate dehydrogenase (EC 1.1.1.8, L-GPD) activity was determined from supernatants of 1-g liver samples homogenized in ice-cold 0.14 M KCL (11) and centrifuged at 4° at 30,000g for 25 min.

Correlation coefficients of relationships were determined and pairs of means were compared by the Student's *t* test (12).

Results. Comparisons of body weights, liver weights, relative liver size, serum free glycerol, and hepatic glycerokinase activity of 75-day-old, nonfasted, male Wistar and BHE rats used in Experiment 1 are shown in Table I. Serum free glycerol ($P < 0.01$) levels and liver glycerokinase activity measured in supernatant fractions ($P < 0.05$) were significantly higher in BHE rats than in Wistar controls. No significant differences were observed in body weight, liver weight, or relative liver size. Correlation coeffi-

cients between glycerokinase activity and serum free glycerol levels or liver weights were not significant.

Table II compares body weights, liver weights, relative liver size, serum free glycerol, and hepatic glycerokinase and L- α -glycerophosphate dehydrogenase activity for 75-day-old, nonfasted Wistar and BHE rats in Experiment 2. Although significant differences were observed in body weights ($P < 0.05$) and liver weights ($P < 0.01$), no differences were noted in relative liver size. Body weights and liver weights in Experiment 2 were also different from those observed in Experiment 1, despite the fact that all animals received similar treatment. Thus, in order to permit comparisons of enzyme activity, results are expressed as per 100 g body weight. Glycerokinase activity measured in crude homogenates in Experiment 2 was also slightly higher than glycerokinase activity measured in Experiment 1.

TABLE I. COMPARISONS OF BODY WEIGHTS, LIVER WEIGHTS, RELATIVE LIVER SIZE,^a SERUM FREE GLYCEROL, AND HEPATIC TISSUE GLYCEROKINASE ACTIVITY OF 75-DAY-OLD, NONFASTED, MALE WISTAR AND BHE RATS IN EXPERIMENT.

Strain	Final body weight (g)	Liver weight (g)	Relative liver size	Serum free glycerol (μ mole/100 ml)	Glycerokinase activity (μ mole α -glycerophosphate produced/min/100 g body weight)
Wistar (6) ^b	248 \pm 9 ^c	10.71 \pm 0.43	4.30 \pm 0.05	3.8 \pm 0.75	2.7 \pm 0.5
BHE (8)	280 \pm 5	11.43 \pm 0.39	4.06 \pm 0.11	7.4 \pm 1.60 ^d	7.6 \pm 1.6 ^e

^a Relative liver size = (liver weight/body weight \times 100).

^b Indicates number of animals in group.

^c Mean \pm SEM.

^d Values significantly different from BHE at $P < 0.01$.

^e Values significantly different at $P < 0.05$.

TABLE II. COMPARISONS OF BODY WEIGHTS, LIVER WEIGHTS, RELATIVE LIVER SIZE,^a SERUM FREE GLYCEROL, LIVER GLYCEROKINASE ACTIVITY, AND LIVER - α -GLYCEROPHOSPHATE DEHYDROGENASE ACTIVITY OF 75-DAY-OLD, NONFASTED, MALE WISTAR AND BHE RATS IN EXPERIMENT 2.

Strain	Final body weight (g)	Liver weight (g)	Relative liver size	Serum free glycerol (μ mole/100 ml)	Glycerokinase activity (μ mole α -glycerophosphate produced/min/100 g body weight)	L- α -Glycerophosphate dehydrogenase activity (μ mole/100 g body weight)
Wistar (12) ^b	228 \pm 4 ^{c,f}	8.83 \pm 0.24 ^c	3.85 \pm 0.02	1.06 \pm 0.15 ^d	6.3 \pm 0.9 ^e	139 \pm 9
BHE (12)	203 \pm 3	7.65 \pm 0.24	3.80 \pm 0.04	4.05 \pm 0.23	9.9 \pm 0.7	141 \pm 6

^a Relative liver size = liver weight/body weight \times 100.

^b Indicates number of animals in group.

^c Mean \pm SEM.

^d Values significantly different from BHE at $P < 0.001$.

^e Values significantly different from BHE at $P < 0.01$.

^f Values significantly different from BHE at $P < 0.05$.

Homogenates are usually preferred for measuring maximum glycerokinase activity since there is less chance of losing activity during preparation (10). Correlation coefficients between glycerokinase activity and serum free glycerol, liver weights, or L- α -glycerophosphate dehydrogenase for Experiment 2 were not significant.

Discussion. The data for the two experiments show that free glycerol levels in the BHE rats remain elevated despite increased glycerokinase activity.

At physiological concentrations, i.e., <1 mM, glycerol entry into the hepatocyte is proportional to the extracellular glycerol concentration and is first order (8, 13, 14). As extracellular glycerol concentration increases, glycerol uptake is inhibited by intracellular α -glycerophosphate. Glycerokinase activity is also inhibited by α -glycerophosphate, and this inhibition is competitive with glycerol (6). High ATP concentrations activate glycerokinase when glycerol concentrations are high (5, 7). Inhibition occurs when concentrations of ADP (8) and AMP (6) are high. Thus, at least two possible explanations exist for the presence of the elevated serum free glycerol observed in nonfasted BHE rats: (1) an insufficient glycerol uptake resulting from competitive inhibition by intracellular α -glycerophosphate, or (2) a reversible movement of unphosphorylated glycerol from the hepatocyte into the blood stream. The presence of strain differences in the amount of α -glycerophosphate produced by the liver homogenates used in the experiment indicates that a potential for increased phosphorylation of free glycerol exists in the BHE rats. This increased enzyme activity does not eliminate this step as the possible source of the serum free glycerol, however, since the assay incubation medium used to estimate glycerokinase activity requires a high concentration of ATP. In an *in vivo* situation, reversible movement of glycerol could result from an insufficient supply of ATP within the hepatocyte. A recent study dealing with hepatic tissue cyclic-AMP levels in the BHE strain alluded to such an insufficiency (15). In this experiment, a rapid decrease in cyclic-AMP levels was noted when the animals were subjected to stress. It was suggested that this situation may have been the

result of the animals' attempts to conserve ATP for energy rather than cyclic-AMP production.

The glycerophosphate that is derived from glycerol can be a direct precursor for gluconeogenesis or as a substrate for glyceride synthesis (14), a process that does not involve L- α -glycerophosphate dehydrogenase. L- α -Glycerophosphate dehydrogenase has recently been implicated in gluconeogenesis from glycerol (13, 14). Unless the enzyme was operating at maximum velocity, the lack of strain differences in this enzyme activity probably indicates that any excess α -glycerophosphate produced by the BHE rat is not being used for gluconeogenesis.

Summary. Serum free glycerol and hepatic glycerokinase and L- α -glycerophosphate dehydrogenase were measured in nonfasted, 75-day-old Wistar and carbohydrate-sensitive BHE rats. Serum free glycerol levels and glycerokinase activity were found to be elevated in the BHE strain. These results suggest that hepatic glycerol utilization by the BHE rat is being determined by factors other than the kinetics of the first glycerol-metabolizing step.

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Vasodilators, Intrarenal Distribution of Blood Flow, and Renal Function in Isolated Perfused Canine Kidneys¹ (39502)

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Introduction. Vasodilator compounds increase the renal excretion of salt ($U_{Na}V$) and water (V) in addition to their effects on hemodynamics (1-3). It is difficult to evaluate *in vivo* the mechanisms by which compounds alter renal physiology since they may affect the kidneys directly or indirectly alter systemic hemodynamics and extrarenal organ functions. In the present investigations, we have attempted to simplify analyses of the actions of seven different vasodilator compounds by measuring their effects on renal hemodynamics and renal function in isolated blood-perfused canine kidneys. The isolated preparation permits control of extrarenal hemodynamics (stroke volume, pulse rate, and perfusion pressure) in the absence of an intact nervous system and extrarenal metabolic or endocrine influences. By eliminating these variables, it is possible to bring out patterns of response to vasodilators which are not easily demonstrable *in vivo*. The vasoactive agents used for the present studies included direct acting vasodilators of natural origin (angiotensin, PGE₂, histamine, and acetylcholine) and several compounds which cause vasodilatation indirectly by altering activities of other naturally occurring vasoactive substances (SQ 20881, saralasin, and nifedipine). One additional vasodilator, enalapril, a polypeptide derived from snake venom, was also included in these investigations for comparative purposes.

Materials and methods. Our method of isolated renal perfusion has been described in previous reports (6, 7). Kidneys and aortic blood perfusate (800 ml) were obtained from male mongrel dogs (15-20 kg) which had received heparin (4 mg/kg) and barbitural anesthesia (65 mg/kg, iv).

Isolated perfusion was instituted within 2 min of nephrectomy using a Water's membrane oxygenator and perfusion system. Electrolytes (sodium, potassium, calcium, magnesium, chloride, phosphate, and bicarbonate) and urea were added to the perfusate at rates to replace urinary losses. Glucose, lactate, and pyruvate were dissolved in a 0.45% saline solution to final concentrations of 2 mg/ml, 9.0 mg/ml, and 0.9 mg/ml, respectively. In addition, this solution contained regular insulin (0.02 units/ml), antidiuretic hormone (2 munit/ml), and creatinine and *para*-aminohippurate (PAH) for clearance purposes. The solution was delivered into the renal venous perfusate at a rate (0.5 ml/min) which maintained the perfusate concentration of glucose in a range of 75-110 mg/dl and which provided sufficient or excess metabolic substrates, insulin, and antidiuretic hormone to the kidney for its energy needs and physiologic functions (8-10). Hemodynamic measurements included renal perfusion pressure (Narco manometer), total renal blood flow (RBF), and the intrarenal distribution of blood flow (radioactive microsphere method of McNay and Abe as adapted for the isolated kidney) (7). By the latter technique, the absolute and percentage of blood flows may be measured to the outer and inner halves of the renal cortex with that fraction of blood flow to the inner half of the canine renal cortex also reflecting medullary blood flow. Parameters of renal functions were assessed by measurement of clearances of creatinine (C_{Cr}), PAH, sodium, and osmolality.

Each vasodilator was infused into the renal arteries of five isolated kidneys during 15- to 30-min clearance periods at times of stable renal function. The rates of infusion were adjusted to increase renal blood flow by approximately 20-40% over control measurements with maintenance of renal

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perfusion pressure at a constant systolic level of 130 mm Hg. The dose range to accomplish this for each vasodilator is specified in the legend to Fig. 1. Hemodynamic and renal functional measurements were compared for the periods prior to and during the infusion of each vasodilator.

Results. Figure 1 and Table I summarize hemodynamic and renal functional data obtained before and during infusions of vasodilator substances into 35 isolated canine kidneys. Each of seven vasodilators produced comparable increases in mean RBF (20 to 40%) at constant renal perfusion pressure. In all cases, no matter which vasodilator was infused, the fraction of RBF which perfused the inner renal cortex and medulla was increased to a greater extent as a result

of vasodilatation than was the fraction of RBF which perfused the outer renal cortex. These data were consistent whether the intrarenal distribution of blood flow was measured by the microsphere technique or by the extraction ratio of PAH (E_{PAH}), which was diminished during the infusion of vasodilators.

In contrast to similar effects of each vasodilator on RBF, renal perfusion pressure, and intrarenal distribution of blood flow, the effects on the glomerular filtration rate (GFR) as measured by C_{Cr} varied widely (Table I). Eledoisin and PGE_2 caused significant reductions of C_{Cr} whereas the effect of bradykinin was insignificant. Infusions of two inhibitors of the renin angiotensin system, SQ 20881 (inhibitor of angiotensin I

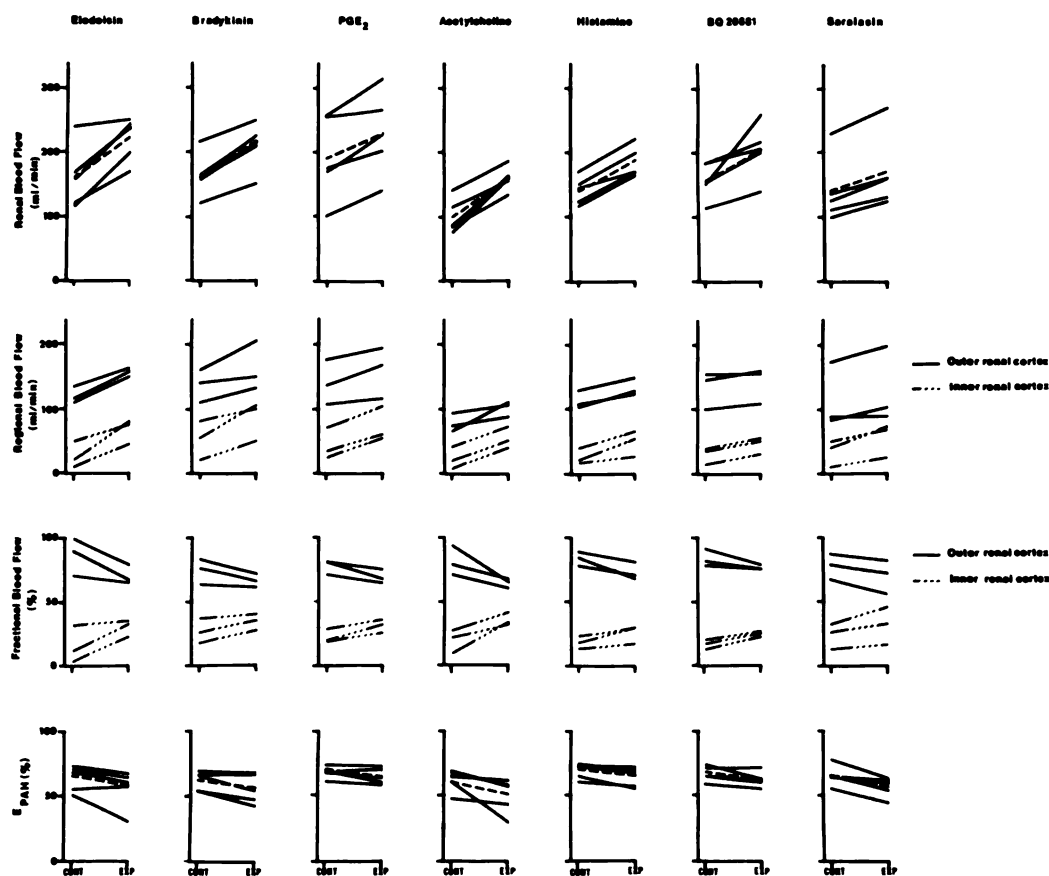


FIG. 1. Effects of vasodilators on renal hemodynamics. Solid lines signify changes in individual kidneys; broken lines signify mean changes. Infusion rates of vasodilators were ($\mu\text{g kg}^{-1} \text{ min}^{-1}$): eledoisin, 0.10–0.40; bradykinin, 0.02–0.09; PGE_2 , 0.01–0.11; acetylcholine, 0.25–5.0; histamine, 0.50–4.60; SQ 20881, 2.50–5.00; saralasin, 2.20–8.10. Note that each vasodilator caused increase in fractional blood flow to inner zone of kidneys.

TABLE I. EFFECTS OF VASODILATORS ON RENAL FUNCTION.^a

C_{Cr} ^b (ml/min)	C_{Cr}/C_{PAH} ^c (%)	C_{Cr}/RBF ^d (%)	FE_{Na} ^e (%)	FE_{H_2O} ^f (%)	$U_{Na}V$ ^g (μ Eq/min)	V ^h (ml/min)
20.5 \pm 3.0	33.3 \pm 4.8	14.8 \pm 2.8	4.0 \pm 0.5	5.6 \pm 1.7	116 \pm 17	0.99 \pm 0.20
13.3 \pm 2.8	19.1 \pm 1.4	6.4 \pm 1.1	2.6 \pm 0.6	5.4 \pm 1.3	54 \pm 16	0.62 \pm 0.13
<0.05	<0.05	<0.05	<0.025	N.S.	<0.01	<0.025
19.0 \pm 0.8	29.4 \pm 3.0	12.5 \pm 1.3	3.6 \pm 1.3	6.8 \pm 2.8	112 \pm 50	1.31 \pm 0.56
16.7 \pm 1.4	21.6 \pm 1.8	7.9 \pm 0.2	4.4 \pm 1.7	9.4 \pm 4.0	128 \pm 54	1.65 \pm 0.78
N.S.	<0.02	<0.025	N.S.	N.S.	N.S.	N.S.
23.7 \pm 3.3	26.7 \pm 5.1	13.6 \pm 2.8	2.9 \pm 0.9	4.4 \pm 1.0	104 \pm 38	0.98 \pm 0.29
18.3 \pm 2.4	20.8 \pm 3.3	8.8 \pm 1.2	2.8 \pm 1.0	5.6 \pm 1.3	79 \pm 29	0.95 \pm 0.24
<0.05	N.S.	<0.05	N.S.	<0.02	N.S.	N.S.
14.4 \pm 2.6	36.0 \pm 4.3	15.8 \pm 3.1	6.5 \pm 0.3	5.9 \pm 0.7	124 \pm 20	0.84 \pm 0.16
16.1 \pm 2.4	23.4 \pm 1.9	10.4 \pm 1.1	16.8 \pm 4.4	16.4 \pm 4.7	332 \pm 69	2.53 \pm 0.57
N.S.	<0.05	N.S.	<0.05	<0.05	<0.05	<0.025
16.3 \pm 1.4	23.9 \pm 2.8	11.7 \pm 1.4	3.6 \pm 0.9	3.8 \pm 0.8	76 \pm 19	0.58 \pm 0.11
17.2 \pm 1.5	19.7 \pm 2.2	9.2 \pm 0.9	2.7 \pm 0.6	3.2 \pm 0.5	61 \pm 12	0.52 \pm 0.06
N.S.	<0.01	<0.01	<0.05	N.S.	N.S.	N.S.
16.6 \pm 0.6	27.1 \pm 2.3	11.0 \pm 0.7	4.4 \pm 1.2	4.0 \pm 1.4	98 \pm 31	0.67 \pm 0.21
16.8 \pm 0.7	20.3 \pm 1.5	9.0 \pm 1.0	4.8 \pm 1.3	5.4 \pm 1.5	124 \pm 35	0.90 \pm 0.25
N.S.	<0.02	N.S.	N.S.	<0.05	N.S.	N.S.
15.9 \pm 2.6	31.3 \pm 4.0	11.4 \pm 1.4	8.0 \pm 1.7	9.2 \pm 1.3	174 \pm 36	1.42 \pm 0.25
16.7 \pm 3.2	24.8 \pm 1.0	10.0 \pm 0.8	8.8 \pm 1.3	10.4 \pm 2.0	212 \pm 69	1.95 \pm 0.78
N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

^a \pm standard errors are compared before (control) and during (experimental) infusions of vasodilators into each of five isolated kidneys. Chance of effects is calculated on basis of Student's paired *t* test. N.S. = not significant.

^b Clearance of creatinine.

^c Clearance of PAH.

^d Renal blood flow.

^e Fractional excretion of sodium.

^f Fractional excretion of water.

^g Secretory rate of sodium.

^h Flow.

g enzyme) and saralasin (competi-
gionist for receptors of angiotensin
ed little change in C_{Cr} . Similarly,
: and acetylcholine did not reduce

The latter agents, if anything,
increase the C_{Cr} , although an in-
number of studies was performed
definite conclusions. Despite the
effects of vasodilators in respect to
ch compound except saralasin re-
ratio of GFR to renal blood flow
(fraction) in the isolated kidney.
ults were obtained by calculating
ion fraction in two ways (C_{Cr}/RBF
sis of the total renal blood flow or
on the basis of clearance of PAH).

tended to reduce the filtration
Iso, but this effect was not signifi-
(< 0.2) relative to the few experi-
ich were conducted, possibly since
s of saralasin may have varied

widely dependent on the state of renin pro-
duction by the individual kidneys.

Salt and water reabsorption and excretion
were affected by the several vasodilators in
different ways (Table I). These differences
could be attributed to the dissimilar re-
sponses of the vasodilators on the GFR and/
or the tubular reabsorption of salt and wa-
ter. Eledoisin caused the most marked re-
ductions in $U_{Na}V$ and V since it diminished
the GFR as it enhanced the tubular reab-
sorption of salt (decreased FE_{Na}). There was
little change in $U_{Na}V$ and V during the infu-
sion of histamine. Small reductions in the
reabsorption of water occurred during infu-
sions of bradykinin, PGE_2 , and SQ 20881.
This effect allowed a slight diuresis to occur
in those cases where these agents did not
reduce GFR to a marked degree. In gen-
eral, FE_{Na} (fractional excretion of sodium)
was affected less by these compounds than

FE_{H_2O} . Acetylcholine differed from all other vasodilators presently investigated since it caused a marked natriuresis and diuresis primarily by its ability to reduce the tubular reabsorption of salt and water. The effect of saralasin on sodium and water metabolism has been inconsistent in our studies to date in the isolated kidney.

Discussion. Present investigations in isolated, perfused kidneys indicate that vasodilators uniformly increase fractional RBF to the inner renal cortex (Fig. 1). This response was obtained consistently in 21 experiments using seven different vasodilators. Our previous studies in isolated kidneys demonstrated an opposite effect of vasoconstrictors to diminish fractional RBF to this same inner zone of the kidney (7, 11). These results indicate relatively greater alterations of vascular resistance in the deep renal circulation compared with the outer renal circulation in response to vasoactive compounds. Although the mechanism for this differential effect is unknown, the fact that all vasoconstrictors tested in the isolated kidney act one way and all vasodilators act another way indicates a common mechanism and suggests that an inherent anatomic feature of the deep renal vasculature might be involved in these responses. It may be pertinent that vascular resistance in the inner renal circulation has been reported to be much greater than in the outer renal circulation (12) as a consequence of the great length of arterioles in the deep renal cortex and medulla (13). Therefore, the possibility is to be considered that these high resistance vessels may be especially responsive to the effects of circulating vasoconstrictors and vasodilators as one explanation for present results.

Although other investigators have infused vasodilator and vasoconstrictor hormones into kidneys *in vivo* without consistent effects on intrarenal blood flow (14, 15), there are several factors to explain these differences. First, it is not possible to control renal perfusion pressure as exactly *in vivo* as can be accomplished in isolated kidneys, and alterations of perfusion pressure influence of the intrarenal distribution of

blood flow (16). Also, vasodilators stimulate compensatory adjustments of other regulatory systems *in vivo*, such as sympathetic nervous responses, renin or prostaglandin release which help determine the intrarenal distribution of blood flow (7, 11). The rates at which vasodilator or vasoconstrictor compounds were infused may be an additional factor. In present studies, doses of vasodilators were infused that caused only moderate increases in RBF. Normally, only a small fraction of the total RBF perfuses the deep cortical and medullary circulations (12, 15). If one infuses vasoactive agents in concentrations to produce marked changes in RBF, then of necessity a large part of the effect must involve the outer renal cortex since the capacity of inner cortical blood flow to change is limited. Thus, large doses of vasoactive compounds may mask a greater relative responsiveness of the deep renal vasculature to compounds, which is apparent only at a lower dose range.

It is of interest that different vasodilators had dissimilar effects on GFR despite comparable effects on other aspects of renal hemodynamics. Such differences can be caused in part by relatively greater effects of specific vasodilators on renal afferent or efferent arterioles to alter glomerular hydrostatic pressure (15). Other mechanisms may be that various compounds affect the glomerular capillary membrane differently to alter the ultrafiltration coefficient or cause different responses in intratubular pressure (15). Although our data demonstrated reductions of the filtration fraction after each vasodilator, indicative of greater efferent than afferent arteriolar relaxation, no additional information was obtained to allow an accurate analysis of why the C_{Cr} was altered differently by the vasodilators which we studied.

Despite comparable effects on RBF and perfusion pressure, the dissimilar patterns in response of $U_{Na}V$ and V to infusions of the different vasodilators are apparent in Table I. Several of these deserve further comment. Thus, the present data demonstrate the potency of acetylcholine relative to other vasodilators in diminishing the tubular reabsorption of salt and water. PGE_2 and

renin had similar effects on salt and metabolism in the isolated kidney. Bradykinin has been shown to release the kidney (17), a part of its effect have been related to an increased of endogenous PGE_2 . It was recently that following inhibition of renin synthesis by indomethacin, renin lost much of its ability to inhibit the renal excretion of free water. The ability of SQ 20881 to increase GFR and $\text{U}_{\text{Na}}\text{V}$ may have been related also to the activity of endogenous bradykinin E_2 since SQ 20881 not only inhibits conversion of angiotensin I to angiotensin II but also inhibits the kininase responsible for the degradation of bradykinin (4). The relatively consistent effects on $\text{U}_{\text{Na}}\text{V}$ were measured from experiment to experiment for each of the vasodilators: saralasin, saralasin has both agonistic and antagonistic effects on angiotensin release which are dependent in part on rates of endogenous renin release (5, 18). Possible inconsistent effects on salt and water balance in various isolated kidneys represent differences in its agonistic or antagonistic actions relative to the level of renin present on by the isolated kidneys.

Summary. Each of the seven different vasodilators (acetylcholine, histamine, bradykinin, saralasin, SQ 20881, deledoisin) infused into isolated blood-perfused canine kidneys at constant perfusion pressure (130 mm Hg) at a flow rate cause 20–40% increases in RBF. Increases of fractional blood flow to outer renal cortex and medulla as determined by radioactive microspheres. Denominator hemodynamic effects of the vasodilators on RBF, perfusion and intrarenal distribution of blood flow. Their effects on GFR, $\text{U}_{\text{Na}}\text{V}$, and V were similar. In particular, deledoisin and saralasin reduced GFR and acetylcholine produced the greatest diuresis and natriuresis. The effect to interfere with the tubular reabsorption of salt and water.

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Regeneration of the Guinea Pig Parotid Gland after 4-hydroxyaminoquinoline-1-oxide-Induced Necrosis (39503)

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4-Hydroxyaminoquinoline-1-oxide (4-HAQO), a presumed proximate carcinogen resulting from the metabolism of 4-nitroquinoline oxide, is carcinogenic in several organs of rodents (1-3). Recently it has been shown that a single iv injection of 4-HAQO induces necrosis of pancreatic acinar cells in rats and guinea pigs (4-7). Furthermore, we have demonstrated that pancreatic acinar necrosis in the guinea pig induced by 4-HAQO is followed by significant regeneration (5, 6). The present study was undertaken to investigate the effect of 4-HAQO on the parotid gland, since the morphology and function of the parotid gland are somewhat similar to those of the exocrine pancreas. We now report that a single iv injection of 4-HAQO in a dose of 22 mg/kg body weight causes profound necrosis of the guinea pig parotid acinar tissue within 48 hr and that the necrosis is followed by active regeneration.

Materials and methods. Randomly bred male guinea pigs weighing between 250-300 g, obtained from Small Stock Industries, Inc., Arkansas, were used in these studies. They were housed in groups of three to four per cage and maintained on guinea pig Purina chow. 4-HAQO (a generous gift from Dr. Elizabeth Weisberger, NIH), dissolved in 0.01 N HCl immediately before use, was injected into the antecubital vein at a dose of 22 mg/kg body weight. The control animals were injected with a corresponding volume of 0.01 N HCl solution. Three to seven animals in the test group and three animals in the control group were sacrificed at 6-hr intervals beginning from 24 hr after injection up to 108 hr. For light microscopy, all the salivary glands were fixed in 10% neutral buffered formalin and embedded in paraffin, and 5- μ m sections

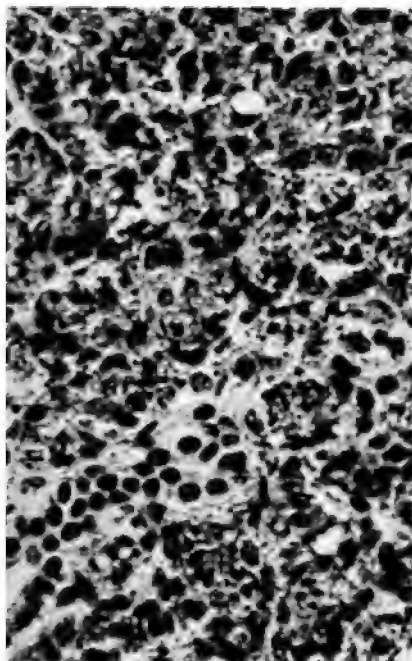
were routinely stained with hematoxylin-eosin stain.

To assess DNA synthesis and cell division in the parotid glands, [³H]thymidine (sp act 53.3 Ci/mole, New England Nuclear Corp., Boston, Massachusetts) uptake was investigated by autoradiography at the light-microscope level. The control and HAQO-treated animals were injected with [³H]thymidine at a dose of 0.4 μ Ci/g body weight ip 1 hr before sacrifice at 6-hr intervals starting from 48 hr. Tissue was processed for autoradiography as described previously (5). Nuclei overlaid with more than five grains were scored as labeled. Approximately 3000 parotid acinar cells from each animal were counted to obtain the labeling and mitotic indices.

Results. A single iv injection of 4-HAQO has induced severe necrosis of the acinar cells of the parotid gland between 24 and 48 hr. The necrosis was followed by regeneration between 54 and 108 hr. The control animals, injected with HCl alone, revealed no evidence of necrosis in the parotid or other salivary glands at any interval.

Necrotic phase. Grossly, the parotid glands of guinea pigs 24 hr after 4-HAQO injection resembled that of controls. However, after 30 hr they were pale and edematous. Histologically, at 24 hr, the lobular and acinar architecture was well preserved. The cytoplasm of the acinar cells was vacuolated with decrease in granularity. An isolated acinar cell showed nuclear pyknosis or necrosis. The interstitial tissue was edematous and devoid of inflammatory reaction. Between 30 and 48 hr there was progressive increase in cellular injury with distortion of lobular architecture (Figs. 1 and 2). An occasional uninvolved acinar cell was present, scattered among the necrotic acinar cells. The excretory ducts of different sizes showed no evidence of cellular damage. There was no histologic evidence of necrosis

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At 30 hr after 4-HAQO injection, the parotid tissue shows necrosis of acinar cells. H & E, $\times 450$.

submandibular or sublingual glands. **Regenerative phase.** At 54 hr, there was loss of the necrotic tissue. Regenerativity was prominent at 54 hr and many damaged acini, as well as newly formed acini, appeared to proceed rapidly. Several mitoses were noted in parotid acinar cells at 54 hr after 4-HAQO treatment. The mitotic count was in animals killed at 72 and 84 hr after 4-HAQO injection (Figs. 3 and 4). Newly formed acinar cells contained a hyperchromatic nucleus and scant cytoplasm (Figs. 4 and 5). Between 60 and 84 hr, newly formed acinar cells showed a fivefold increase in the amount of cytoplasm. No mitotic activity was evident in the duct system.

Effect of 4-HAQO administration on incorporation of [^3H]thymidine in the parotid gland is shown in Fig. 3. A progressive increase in the percentage of labeled cells was noted between 54 and 72 hr; the degree of labeling of acinar cells was 10% at 72 hr. Thereafter, a gradual decline in nuclear labeling of parotid acinar cells was noted. No significant nuclear label-

ing was encountered in the cells lining the ducts.

Comment. The data presented here demonstrate that a single iv injection of 4-HAQO causes necrosis of the parotid gland in the guinea pig between 24 and 48 hr and that the necrosis is followed by marked regeneration and restitution of the acinar tissue. The 4-HAQO-induced necrosis of the salivary tissue in the guinea pig was limited to the parotid gland only; the submandibular and submaxillary salivary glands remained unaffected. The necrotic effect of 4-HAQO in parotid glands appeared similar to that induced by this compound in exocrine pancreas (5, 6). In the pancreas, as well as in the parotid gland, the maximum necrosis occurred by 48 hr after the injection of this compound. The mechanism by which 4-HAQO causes necrosis of the parotid acinar tissue remains to be elucidated. In the pancreas, the necrogenic effect of 4-HAQO has been considered to be caused, possibly, by two mechanisms: (1) the ability of this compound to bind to DNA, leading to interference of DNA-directed RNA synthesis, and (2) enhanced uptake of this carcinogen by the pancreatic exocrine tissue (7,

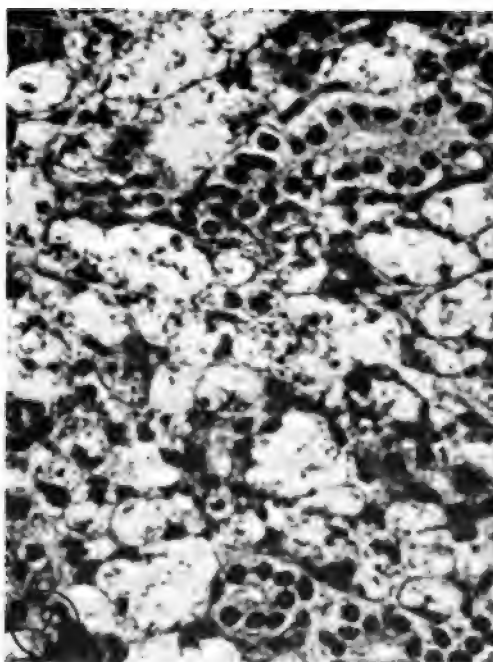


FIG. 2. At 48 hr after 4-HAQO, The necrosis of acinar tissue was marked. H & E, $\times 450$.

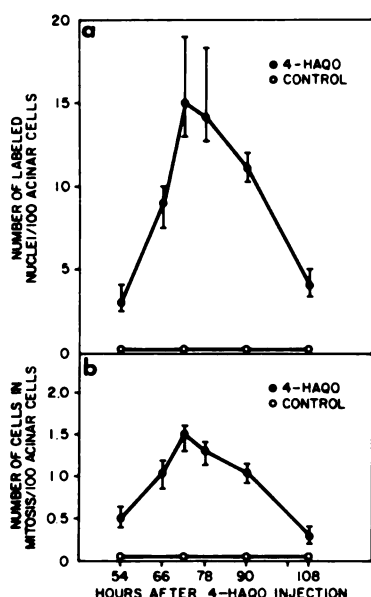


FIG. 3. The effect of 4-HAQO injection on [3 H]thymidine acinar cell nuclear labeling between 54 and 108 hr in autoradiographic studies. The effect of 4-HAQO injection (a) on the incorporation of [3 H]thymidine into the parotid acinar cell nuclei as determined by nuclear labeling in autoradiographic studies, and (b) on the number of mitoses in acinar cells. The values are mean \pm SEM for three animals.

8). Presumably these two mechanisms may be in operation in the 4-HAQO-induced necrosis of the parotid epithelium. The studies of Iqbal *et al.* (9) have shown that 4-HAQO induces DNA damage in the guinea pig pancreatic slices *in vitro* and that repair of the damaged DNA occurs fairly rapidly. Additional studies are needed to ascertain if 4-HAQO induces DNA damage and DNA repair in the parotid gland. The concurrent occurrence of necrosis of the exocrine pancreatic and parotid tissues in the guinea pig following iv injection of 4-HAQO may be due to greater affinity of these exocrine tissues to concentrate this chemical. There is, however, very little information available regarding the uptake and metabolism of carcinogens by the exocrine glands.

The regenerative changes in the parotid gland which followed the 4-HAQO-induced necrotic phase appeared similar to those described in the pancreas after a single iv dose of this carcinogen (5, 6). Regeneration and repair of parotid acinar tissue occurred between 54 and 108 hr after 4-HAQO injection.

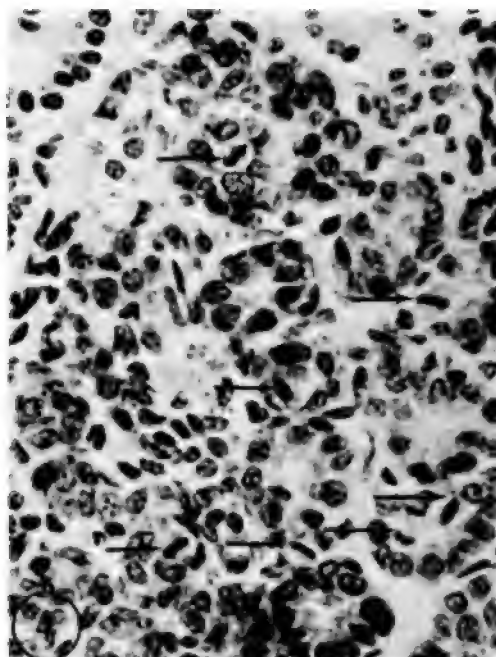


FIG. 4. At 72 hr after 4-HAQO injection, numerous mitoses (arrows) are seen in the parotid acinar cells. H & E, $\times 560$.

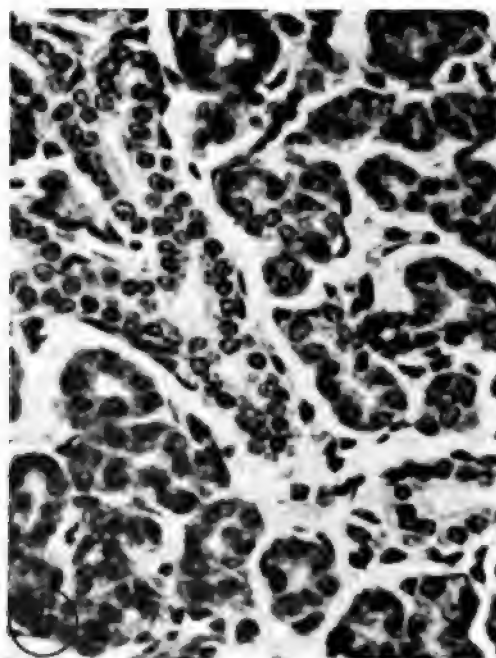


FIG. 5. At 96 hr after 4-HAQO injection, the newly formed acini are lined by low cuboidal cells containing hyperchromatic nuclei and moderate cytoplasm. H & E, $\times 450$.

However, even at 108 hr, the newly formed acini were small and appeared functionally immature in view of the diminished basophilic granular appearance of the acinar cells. Increased [^3H]thymidine labeling of acinar cells, as well as increased mitotic activity of parotid acinar cells resulting from 4-HAQO treatment, are clear indications that acinar cells, usually "nondividing tissue" (10), in which mitoses are rare, can be stimulated to proliferate actively by experimental manipulation. Although the precise mechanism of 4-HAQO-induced parotid acinar cell regeneration is not clear, it is reasonable to assume that a proliferative response is triggered by the necrosis and loss of acinar tissue. The regenerative response of the parotid gland observed in the present studies is comparable to that described in the rat parotid gland after ethionine administration (11, 12) or after x-ray irradiation (13), both of which are associated with necrosis of acinar tissue. In contrast, however, the studies of Barka (14) have unequivocally demonstrated that the undifferentiated parotid acinar cells can be stimulated to proliferate by the administration of isoproterenol without antecedent necrosis.

Summary. A single iv injection of 4-hydroxyaminoquinoline-1-oxide (4-HAQO) in a dose of 22 mg/kg body weight induced necrosis of parotid acinar tissue in guinea pigs within 48 hr. After the necrotic phase, marked regenerative activity of the parotid cells was noted. [^3H]Thymidine autoradiographic studies revealed a labeling index of 15% cells in the parotid gland at 72 hr after 4-HAQO injection. A significant increase in the mitotic activity of the parotid

acinar cells was also evident at 72 and 78 hr after 4-HAQO administration. The acinar cell repair, as well as formation of the new acini, were complete at 108 hr. It is clear from the studies that 4-HAQO, a pancreaticototoxic agent and a potent carcinogen, also causes necrosis of the parotid acinar tissue, which is followed by regeneration and restitution.

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Suppression of Postnatal Changes in Spermatogenesis, Size, and Nucleic Acid Content of Rat Testis by Chronic Administration of Isoproterenol (39504)

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Introduction. Chronic administration of large doses of the catecholamine isoproterenol can evoke marked proliferative activity in organs that normally exhibit low mitotic levels (1, 2). However, where mitosis is normally high, as in salivary glands of preweanling rats (3) or in intestinal mucosa of adult mouse (4), proliferation is not further increased but is either not affected at all (5, 6) or inhibited somewhat (7). These results suggest that isoproterenol cannot induce mitosis in tissues that are already dividing rapidly. However, the number of systems on which this effect has been examined is limited. Accordingly, it was considered important to examine the effects of chronic administration of isoproterenol on yet another dividing tissue (8). Testis was considered especially useful for this purpose, for while both the immature and mature organs exhibit high levels of mitosis, they also exhibit different levels of maturation (8). Consequently, this system can be used to distinguish between effects of isoproterenol on normal mitotic activity of the tissue and the influence exerted by degree of tissue maturation on such effects.

Materials and methods. Male Long-Evans rats, ranging in age from 10 days to 4 months, were maintained on lab chow and water *ad lib.* until just before sacrifice. A regimen of twice-daily ip injections of isoproterenol (ISO) in a dose previously shown to induce DNA synthesis (15 mg/kg) (2-6) was initiated 8-9 days before sacrifice of half of the males of each litter; the other half of the litter was untreated or injected with saline only. The age at which drug administration was initiated varied, so that groups receiving the drug for 8-9 days were 28, 35, 42-45, 50-51, and 4 months old at the end of the period of drug treatment. The drug did not significantly modify weight of the animals even though it was used in pharma-

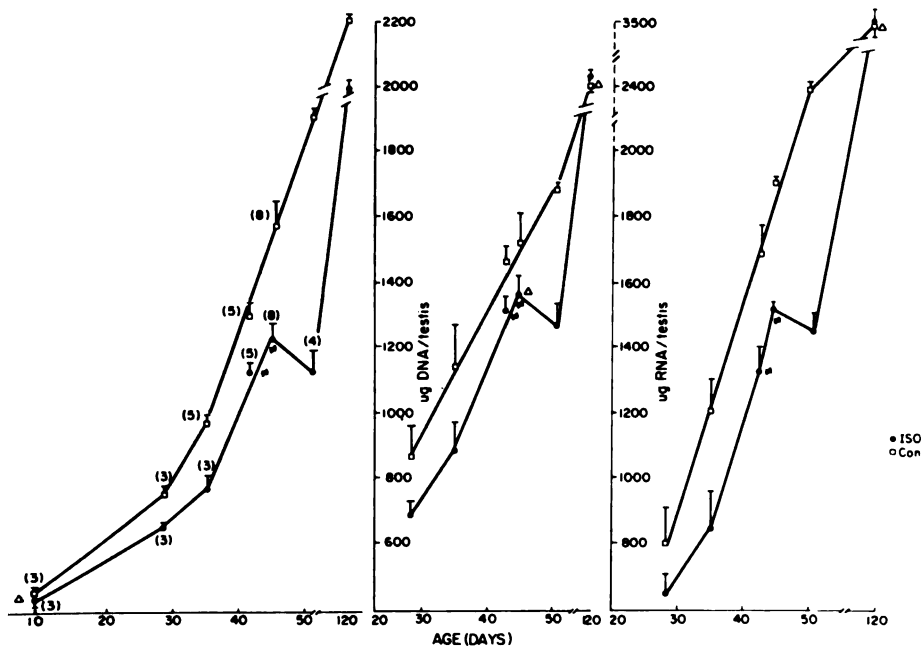
cological doses. At the end of the period of drug administration, animals were anesthetized with 1% Nembutal and both testes were removed and individually weighed on a torsion balance; one was placed in ice-cold 0.4 N HClO₄ for subsequent nucleic acid extraction and the other was minced in isotonic Na-citrate with hyaluronidase (150 N.F. units/10 ml) for subsequent analysis of spermatogenesis. DNA was determined by the Burton modification of the diphenylamine reaction (9). RNA was determined by the orcinol reaction (10). The course of spermatogenesis was followed using the technique of Evans (11) with modifications as suggested by Schleirmacher (12). The minced testis preparation was placed on a magnetic stirrer for 10 min to release cells from the tubules. Following this, the suspension was allowed to stand a few minutes to allow the remaining tubules and connective tissue to settle to the bottom of the beaker. The supernatant was removed and centrifuged at 660 rpm, and the spermatogonia, spermatocytes, secondary spermatocytes, spermatids, and mature sperm were thus separated. These were treated with 3 ml of hypotonic (1% wt/vol) sodium citrate solution for 12 min, again centrifuged, and the supernatant was discarded. The cell pellet was suspended in a 3:1:0.025 absolute ethanol:acetic acid:chloroform fixative mixture. This suspension was again centrifuged and resuspended in 0.5 ml of fresh fixative.

The microscope slides were made by splashing a few drops of this suspension onto slides which had been chilled in distilled ice water. After drying, the slides were stained with Giemsa and mounted with Permount. Five slides were made from the testes of each rat, and 500 cells were counted. These were separated into appropriate phases of mitosis or meiosis on the basis of their chromosomal configuration.

as observed were spermatogonia; zygotene, pachytene (L-Z-P); diakinesis (D-D); metaphase I (M I); and mature sperm.

From the data in Fig. 1, it is clear that between 10 and 51 days of age, weight, DNA, and total RNA of rat testis increased progressively. Testis weight increased approximately 55% at weekly intervals. Total RNA increased about 55% between 28 and 35 days of age, and DNA increases thereafter were only less than this (approximately 45%). On the other hand, DNA increased about 55% between 28 and 35 days of age and RNA increases thereafter were only less than this (approximately 45%). The increase was significantly less ($P < 0.05$) between 35 and 42 days of age, and between 42 and 50 days of age, only a 14% increase appeared. The RNA/DNA ratio was less than 1 at 28 days of age, 1 at 35 days of age, and greater than 1 thereafter; at 51 days of age it was 1.47.

Chronic administration of isoproterenol for periods of 7-8 days did not prevent age-related changes in weight and nucleic acids but the course and characteristics of the changes were greatly altered. Thus, while weight and nucleic acid content of testes of isoproterenol-treated rats increased between 28 and 42 days of age, the magnitude of the increase in each age (28, 35, and 42 days) was less than that exhibited by testis of untreated littermates (Fig. 1). Thus, at 28 days of age, the weight of testis in isoproterenol-treated rats was 27% less than that of the testis of untreated littermates, and DNA and RNA levels were each 20% less than those of untreated animals. The differences at 35 days were somewhat greater: weight was 36% less, and DNA and RNA were from 25-30% less. The smallest difference was seen at 42 days, when weight and RNA of testis of isoproterenol rats were about 20% less than that of untreated rats, and DNA was only 10% less. The RNA/DNA ratio of testis of isoproterenol treated rats



Postnatal changes in weight, DNA, and RNA content of testes of untreated rats (Con) and of rats injected with isoproterenol (ISO). Each point is the mean \pm SE obtained from three to eight animals; errors of littermates were untreated or injected with 15 mg/kg of isoproterenol, ip, twice daily for 7-8 days. Nucleic acids (DNA and RNA) are expressed as micrograms per testis, and wet weight of testis in milligrams. In one age to the next that were not of statistical significance ($P > 0.05$) are designated with #; between Con and ISO-treated animals, at each age, were statistically significant ($P < 0.05$) except indicated with Δ .

TABLE I. POSTNATAL CHANGES IN NUMBER OF MATURE SPERM AND DISTRIBUTION OF MITOTIC AND MEIOTIC CELLS OF SEMINIFEROUS TUBULES OF RAT TESTIS, AND MODIFICATION BY CHRONIC ADMINISTRATION OF ISOPROTERENOL.^a

Age (days)	Number of rats	Percentage spermatogonia ^b (mitotic)	Percentage				Total percentage of meiotic cells ^c	Number of sperm
			L-Z-P	D-D (meiotic)	M I	M II		
Control								
28	4	55.4 ± 0.9	36.5 ± 0.6	6.9 ± 0.05	0.8 ± .17†	0.2 ± 0.2	45.6	0
35	6	45.7 ± 1.0	49.1 ± 1.0	4.3 ± 0.4	0.6 ± 0.05	0.03 ± 0.03	54.3	0
42	14	38.2 ± 0.8	54.7 ± 1.1†	6.0 ± 0.4	0.8 ± 0.1	0.01 ± 0.01	61.8	146 ± 24
50	5	24.7 ± 0.8	55.5 ± 0.4	18.3 ± 0.8	1.2 ± 0	0.35 ± 0.1	75.3	454 ± 16
Adult	5	37.4 ± 1.3	53.5 ± 2.3	8.4 ± 1.5	0.7 ± 0.1	0	62.6	1103 ± 60
ISO-treated								
28	6	55.8 ± 1.1*	37.3 ± 0.9*	5.6 ± 0.2	0.9 ± 0.1	0.2 ± 0.1	44.2	0
35	6	52.3 ± 0.5	44.2 ± 0.7†	3.4 ± 0.3	0.4 ± 0.1†	0	47.7	0
42	12	43.5 ± 1.2†	46.6 ± 1.6†	7.7 ± 1.3*	0.5 ± 0.1	0.09 ± 0.06	56.5	54 ± 35
50	5	44.1 ± 1.2	44.8 ± 1.6	8.7 ± 0.2	1.5 ± 0.2*	0.7 ± 0.2*	55.9	141 ± 46
Adult	5	35.4 ± 2.6*	52.1 ± 1.2*	11.6 ± 3.5*	0.9 ± 0.1*	0.04 ± 0.04*	64.6	923 ± 84*

* Values are means ± SE. Changes from one age to the next that are not of statistical significance ($P > 0.05$) are designated with †; differences between control (untreated) and ISO-treated (twice daily ip injections of isoproterenol for 7-8 days) animals, at each age, were statistically significant ($P < 0.05$) except when designated with *. Stages as follows: spermatogonia (mitotic); L-Z-P, leptotene-zygotene-pachytene (meiotic); D-D, diplotene-diakinesis (meiotic); M I and M II, metaphase I and metaphase II (meiotic); sperm (mature sperm, and values are number of sperm counted in a field of 500 spermatogenic cells).

^b Number of cells at each stage expressed as percentage of total number of spermatogenic cells (excluding sperm).

^c Value in each case is sum of L-Z-P, D-D, M I, and M II percents.

was the same as that for controls at 28 days of age and 35 days of age; however, unlike the untreated rats, no change occurred thereafter and RNA/DNA was between 1.0-1.1.

Although administration of isoproterenol to adults for 7-8 days caused a statistically significant decrease in weight of testes ($P < 0.05$), the change was small in magnitude (7%), and the other parameters measured were not affected at all (Fig. 1).

As age of the animals increased, there was not only the prominent shift in pattern of increase in cell size and number but there was also a shift in the events of spermatogenesis. Isoproterenol effected changes in the course of spermatogenesis that were consistent with effects of this agent on size and nucleic acid content of the testes. The data in Table I show these effects, and proportions of cells found in seminiferous tubules for each age are shown for control as well as ISO-treated animals. As an untreated (control) animal aged, the number of cells found in the spermatogonial stage progressively decreased, and, at 28 days, this number represented 55% of all spermatogenic cells; at 35 days of age, 46%; at 42 days, 38%; and at 50 days of age, 25%. The change from one age to the next was in each case statistically significant ($P < 0.05$). As

the percentage of spermatogonial cells decreased, the percentage of meiotic cells increased. Thus, at 28 days, meiotic cells comprised approximately 45% of all spermatogenic cells; at 35 days, 54%; and at 50 days, 75%. The distribution of cells at each meiotic stage also changed with increasing age of the animals and, for example, at 28 days of age, about 37% of the total spermatogenic cells were in L-Z-P stages of meiosis, and by 50 days of age, 56% were.

Mature sperm were not found as a consequence of spermatogenesis before 42 days of age, when approximately 142 sperm were found in a field of 500 spermatogenic cells; by 50 days, there was more than a threefold increase in this number (Table I).

In rats 21 days old at the time of initial injection of isoproterenol, the number of spermatogonial cells was not different from that of untreated rats, and approximately 56% of all spermatogenic cells were in the spermatogonial stage after 7 days of ISO treatment (Table I). As with untreated rats, the number of spermatogonia decreased with increasing age of the animals, but the extent of the decrease was much less. Thus, a statistically significant decrease ($P < 0.05$) was not observed until 42 days of age, at which time the number of spermatogonia had decreased to 44%. No further decrease

at 50 days. Here also, the number entering meiosis increased with age, but the magnitude of the age-related changes was much less than that exhibited in controls. Thus, at 28 days of age, 44% of spermatogenic cells were in meiosis; this percentage did not change until 42 days of age when about 57% were in meiosis and remained at 57% at 50 days (Table I).

The age-related changes in the percentages of cells at each meiotic stage were seen in isoproterenol-treated testes also. However, only a few of the changes were significant. For example, the percentage of spermatogenic cells in L-Z-P phases of spermatogenesis was about 37 at 28 days of age, increased to 44 at 35 days, but remained at that level thereafter (Table I). Generally, the number of meiotic cells in the tubules of isoproterenol-treated rats was, for each age except 28 days, less than the number found in tubules of untreated rats.

The first sperm were first observed at 42 days of age in ISO-treated animals, the age at which mature sperm were first found in the testis of untreated animals. However, only 54 ± 35 were counted in a 500 tubular cells, in contrast to a value 100 times as great in the controls.

In the adult, the percentage of cells in the spermatogonial stage was about 37, 63% were in meiosis, and about 1100 mature sperm were consequently seen. Isoproterenol did not cause any statistically significant changes in any of these values (Table I).

Discussion. Growth and differentiation of spermatogonia normally continue postnatally (8, 13), and in isoproterenol is chronically administered to the immature rat, both processes are greatly retarded. On the basis of measurements of nucleic acids and weight, it is apparent that isoproterenol caused a decrease in cell number of rat testis at each age (28–50 days) examined. In the immature rat (28 days old), the magnitude of the decrease in DNA, RNA, and weight was the same, and the RNA/DNA ratio remained at 1. Thus, only cell number, and not cell size, was affected by the drug. At older ages also (42 and 50 days), cell number but not cell size, was reduced by isoproterenol. From examination of the

effects on spermatogenesis, the drug apparently acts to suppress cell proliferation by inhibiting the mitotic activity of spermatogonial cells. The normal age-related decrease in percentage of spermatogonial cells was inhibited by the isoproterenol and, as a consequence of this inhibition, fewer cells entered into meiosis. However, the distribution of cells among the various meiotic phases was not much modified by the isoproterenol regimen, and usually only the L-Z-P phases reflected the decrease in number of spermatogonia entering meiosis. Ultimately, the number of mature sperm formed also reflected the inhibitory influence exerted by isoproterenol on the spermatogonia, and the number in isoproterenol-treated rats was only one-third that of the control animals at 42 and 51 days of age.

The suppression of spermatogenesis was accompanied by a general suppression of maturation. For example, in preliminary experiments, it was found that the testosterone level in blood of a 42-day-old untreated male was 40/ng%, but no testosterone was measured in blood of the isoproterenol-treated littermates.

The inhibitory effects of the isoproterenol on testes were limited to the testes of immature animals. No significant changes in testes weight, DNA, or RNA were observed when adult animals were chronically injected with isoproterenol. Inhibitory effects of isoproterenol on cell proliferation have been found in salivary glands also, but here also only the undeveloped (15), rapidly dividing glands of the immature rat exhibit such a response (7), and the fully developed and mitotically quiescent glands of the adult animal (or even of older postnatal rats) (16) show a pronounced proliferative activity following isoproterenol administration (1, 2). The dividing cells of the fully developed intestinal mucosa of adult rats also show no response to isoproterenol (5, 14). Thus, the inhibitory effects on mitosis appear to be associated with already rapidly dividing systems, but only those of undifferentiated organs. The mechanism of these effects remains to be delineated.

Summary. Chronic administration of large doses of the catecholamine isoproterenol to immature rats suppressed the postna-

tal changes in testis size and spermatogenesis that normally occur. Nucleic acid content (total DNA and RNA) was reduced from controls at each weekly interval examined. With increasing age there is normally a decrease in number of spermatogonia observed in seminiferous tubules, and at 28 days of age, 56% remain, but by 50 days, only 25% remain. This change is altered by treatment with isoproterenol, and while the number at 28 days is the same, the number remaining at 50 days had decreased to a much smaller extent so that 44% remained. Isoproterenol thus greatly inhibited spermatogenesis, and the number of cells in meiotic stages and number of mature sperm formed were also greatly decreased from control. While mature sperm were first found in untreated and ISO-treated animals at the same age (42 days), the number in ISO-treated rats was only one-third that of controls. This same order of reduction remained evident at 50 days also. Treatment of adults with isoproterenol for 7 days did not alter nucleic acid content, course of spermatogenesis, or production of mature sperm.

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Kinin-Destroying (Kininase) Activity of Cultured Rodent Fibroblasts L-929^{1, 2} (39505)

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Extracts of a cultured cell line of rodent fibroblasts, L-929, were reported to have kinin-forming activity when incubated at pH 7.4 with kininogen substrate from rat aorta or Murphy-Sturm lymphosarcoma (1). The kinin-forming acid protease was purified and further characterized with respect to pH optimum, cellular localization, molecular weight (2). While the fibroblast preparation did not contain kininogen substrate, it did have kinin-destroying activity that was inactivated at acid pH as well as by 1,10-phenanthroline (1). In view of the possible involvement of the vasoactive system in the growth and development of transplanted tumors containing considerable fibroblast networks (3), the nature of kinin-destroying activity of fibroblasts was studied further and forms the basis of this report.

Methods. Cell culture and cell extract preparation. Mouse fibroblasts L-929, obtained from Microbiological Associates (Bethesda, Maryland), were grown for 4 days in 250 ml bottles in minimum Eagle's medium containing 10% fetal calf serum as described previously (1). The cells were detached from the bottle wall at the time of passage, suspended in minimum Eagle's medium, the cell number was determined by microscopic examination, and the cell suspension was centrifuged in a refrigerated centrifuge at 3500 rpm. The cells then were washed in physiologic saline and disrupted by freeze-thaw technique repeated three times. Aliquot volumes of 0.2–0.5 ml of cell extract, approximating 6×10^6 cells, were used for the assays.

Kinin-destroying activity. Fibroblast suspensions (0.2–0.5 ml) were incubated at 37°

with 0.5 ml of synthetic bradykinin (1×10^{-6} g, Sandoz, Hanover, New Jersey) dissolved in 0.05 M phosphate buffer, pH 7.4. Additional buffer was added to 1 ml when indicated, and at 1-, 3-, and 5-min intervals, 1 mg of 1,10-phenanthroline was added to individual incubation mixtures to terminate the reaction. One hundred-microliter aliquots were studied for residual bradykinin activity on the isolated rat uterus muscle preparation perfused at room temperature with modified Tyrodes solution (4). Control samples contained only buffer and synthetic bradykinin. The 10% fetal calf serum was prepared commercially by heating for 1 hr at 56°. Heating of a carboxypeptidase N preparation for 5 min at 56° was reported to decrease the kinin-destroying activity by 80% (5). Incubation of 0.5 ml of the 10% fetal calf serum with 1×10^{-6} g of bradykinin confirmed the fact that the serum per se did not have any kinin-destroying activity. Throughout the study, synthetic bradykinin was used as the reference standard.

pH Profile. The pH profile of the kinin-destroying activity was determined by incubating 0.5 ml of the cell extract with 1×10^{-6} g of bradykinin in 0.5 ml of various buffers ranging in pH from 4.0 to 10.0. The mixtures were incubated for 5 min at 37°, and the reaction was terminated by the addition of 1 mg of 1,10-phenanthroline. The residual kinin activity was assayed on the isolated rat uterus, and the percentage loss of smooth muscle stimulating (kinin) activity was calculated by the following formula:

$$\frac{\text{Activity}_{\text{standard}} - \text{Activity}_{\text{experimental}}}{\text{Activity}_{\text{standard}}} \times 100.$$

Time and concentration study. The time course for kinin destruction at pH 7.0 and 37° was studied by incubating the cell extract-bradykinin mixture for 1-min time intervals up to 5 min. The effect of increasing

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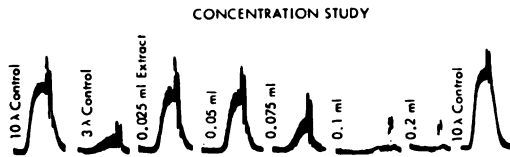


FIG. 1. Smooth muscle-stimulating activity of incubates containing 1×10^{-6} g of synthetic bradykinin and increasing volumes of fibroblast cell extracts (0.025–0.2 ml). Control activity of $10\times$ and $3\times$ bradykinin (following incubation for 5 min with phosphate buffer) is shown.

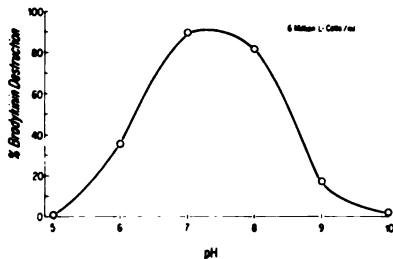


FIG. 2. pH Profile of kinin-destroying (kininase) activity of fibroblast cell extract (0.5 ml) incubated with 1×10^{-6} g of synthetic bradykinin for 5 min at 37° . The reaction was terminated by the addition of 1 mg of 1,10-phenanthroline.

volumes of cell extract on kinin destruction (from 0.02 to 0.20 ml) also was studied following 5-min incubation with synthetic bradykinin (1×10^{-6} g) at pH 7.0 and 37° .

Inhibitor study. The following natural and synthetic inhibitors were studied for their effect on the kinin-destroying activity of the fibroblast cell extract: aprotinin (Trasylol, Farbenfabriken Bayer AG, Leverkusen, Germany), soya bean trypsin inhibitor (SBTI, Worthington Labs, Freehold, New Jersey), lima bean trypsin inhibitor (LBTI, Worthington), heparin (Pan-Heparin, Abbott Labs, Chicago, Illinois), epsilon amino-caproic acid (EACA, Lederle Labs, Pearl River, New York), 4-aminoethylcyclohexane-1-carboxylic acid (AMCHA, Lederle Labs), 2,3-dimercaptopropanol (BAL, Cal Biochem, Los Angeles, California), and 1,10-phenanthroline (Cal Biochem). Increasing concentrations of inhibitor in 0.1 ml of volume were added to 0.2 ml of cell extract and 0.2 ml of phosphate buffer, pH 7.0. The cell extract-inhibitor preparations were allowed to stand for 5 min at room temperature, and then 0.5 ml of bradykinin

(1×10^{-6} g in a phosphate buffer) was added. The mixture was agitated and the tubes were placed in a 37° water bath for an additional 5 min. The reaction was stopped by placing the tubes on ice, and the residual kinin in 100- μ l aliquots was assayed on the isolated rat uterus. Control tubes contained the cell extract, buffer, and synthetic bradykinin. The extent of inhibition was recorded as percentage of residual kinin activity compared to the standard control tubes.

Cell fractionation. Ultracentrifugal cell fractionation studies were carried out to identify the subcellular site of the kininase activity (6, 7). Fibroblast cells (60×10^6) were suspended in 10 ml of 0.25 M sucrose- 1.8×10^{-4} M CaCl_2 solution. The suspension was frozen and thawed 10 times to disrupt the cells and spun at 600g for 10 min to obtain the sedimented nuclear fraction. The supernatant yielded a mitochondria pellet fraction when centrifuged at 10,000g for

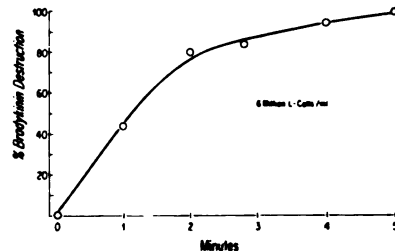


FIG. 3. Time course of kinin-destroying activity following incubation of 0.2 ml of fibroblast cell extract (1.2×10^6 cell equivalent) with 1×10^{-6} g of synthetic bradykinin at 1- to 5-min time intervals at 37° and pH 7.0.

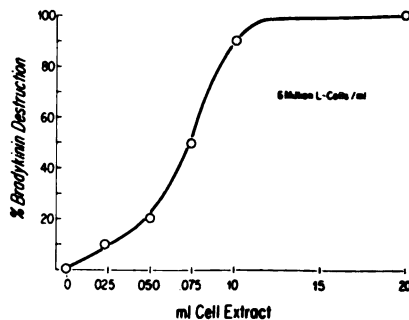


FIG. 4. Effect of increasing concentrations of fibroblast cell extract on the destruction of synthetic bradykinin (1×10^{-6} g) following incubation for 5 min at 37° and pH 7.0.

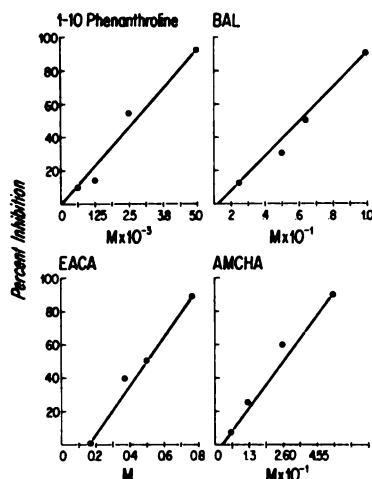


FIG. 5. Effect of 1, 10-phenanthroline, 2,3-dimercaptopropanol (BAL), epsilon amino caproic acid (EACA), and 4-aminoethylcyclohexane-1-carboxylic acid (AMCHA) on the kinin-destroying activity of fibroblast cell extracts. Two-tenths of a milliliter of extract was incubated with increasing concentrations of inhibitors for 5 min, to which was added thereafter 1×10^{-6} g of bradykinin for an additional 5 min.

TABLE 1. CELLULAR LOCALIZATION OF KININ-DESTROYING ACTIVITY OF L-929 FIBROBLAST CELLS.

Cell fraction	Total protein (%)	Kininase activity (%)
Whole cell homogenate	100	100
Nuclear (600g)	12.3	20.0
Mitochondrial (20,000g)	4.9	5.0
Microsomal (100,000g)	1.9	0.0
Soluble Protoplasmic (100,000g)	52.0	64.2

30 min and a microsome fraction after centrifugation at 100,000g for 60 min. The remaining supernatant constituted the soluble protoplasmic protein fraction. Cell fraction aliquots of 0.2–0.4 ml were incubated with 1×10^{-6} g of bradykinin to determine the kininase activity as described above.

Results. Kinin-destroying activity of fibroblast extracts. Figure 1 shows the smooth muscle stimulating responses of the incubation mixtures containing 1×10^{-6} g of bradykinin with increasing volume aliquots of fibroblast extracts. The incubations were carried out at pH 7.4 for 5 min at 37°. The incubation mixtures demonstrated increased kinin-destroying activity, while control tubes containing only bradykinin and buffer

retained undiminished kinin activity after the 5-min incubation period.

pH Profile. Optimum kinin-destroying activity occurred at a neutral pH of 7.0–7.4, Fig. 2, while no kinin-destroying activity was observed at a pH of 5 or lower and at pH 10.0. Thus, activity ranged over a pH of 6.0–9.0. Residual kinin activity in the bradykinin–extract incubation mixtures was compared with control tubes containing bradykinin and buffer and incubated under the same time and pH conditions.

Time and concentration study. Incubation of 0.2 ml of the fibroblast cell extract with 1×10^{-6} g of bradykinin at 37° and pH 7.0 for increasing time intervals resulted in total destruction of the kinin activity within 5 min, Fig. 3. Fifty percent of the kinin activity was destroyed following 1.2 min of incubation whereas almost 80% was destroyed after 2 min. Increasing concentrations of fibroblast extract (0.02–0.2 ml) resulted in increased kinin destruction when incubated with 1×10^{-6} g of bradykinin for 5 min at 37° and pH 7.0, Fig. 4. An aliquot of 0.07 ml of extract caused 50% destruction, while 0.1 ml destroyed 90% of the kinin.

Inhibitor study. The inhibitory profiles of four agents that inhibited the kinin-destroying activity are represented in Fig. 5. The agents included the classical kininase inhibitor 1,10-phenanthroline (ID_{50} , 4.375×10^{-3} M); BAL (ID_{50} , 0.64×10^{-1} M); and two weak inhibitors, EACA (ID_{50} , 0.5 M) and AMCHA (ID_{50} , 0.238 M). At the highest doses studied (indicated in the parentheses), the following agents had no effect on the fibroblast kinase activity: aprotinin (1000 units), soya bean trypsin inhibitor (10 mg), lima bean trypsin inhibitor (10 mg), and heparin (2000 units).

Cell fractionation. Ultracentrifugal cell fractionation showed the soluble protoplasmic protein fraction to contain 64.2% of the kininase activity, while the nuclear portion had 20% and the mitochondria fraction had only 5%, Table 1. The microsome fraction did not contain any activity. Approximately 90% of the total kinin-destroying activity was recovered from these cell fractions.

Discussion. This mouse fibroblast cell line was shown previously to have an acid pro-

tease capable of forming kinins from suitable substrates (1). Purification and further characterization of this enzyme have been achieved (2). The present study has focused on the kinin-destroying activity of this fibroblast cell line (1). This activity was shown to have a pH optimum at neutrality, similar to the activity reported in extracts of rabbit and human leukocytes (8, 9), kidney (10, 11), rat liver (12), and rabbit brain (13).

Studies with the inhibitor suggest that the fibroblast kinin-destroying activity is metal dependent. Both 1,10-phenanthroline, an effective metal-dependent kininase inhibitor (11), and 2,3-dimercaptopropanol (BAL) blocked the fibroblast kinin-destroying activity in a dose-dependent fashion. BAL has been reported to inhibit rat plasma kininase activity *in vitro* (14). The proteinase inhibitor aprotinin and the soya and lima bean trypsin inhibitors were not effective against the fibroblast kinin-destroying activity similar to previously published reports with activities from other tissue sources (15). Aprotinin did inhibit the kininase activity of rabbit brain (16) and rabbit granulocyte extracts (17). The synthetic agents EACA and AMCHA were found to be very weak inhibitors of the fibroblast kinin-destroying activity. EACA, at concentrations of 3×10^{-3} M, did inhibit the kinin-destroying activity of human plasma but not that of hemolyzed human red blood cells or guinea pig serum (15).

The major portion of the kinin-destroying activity resided in the soluble protoplasmic protein fraction of the fibroblast cell (obtained after centrifugation at 100,000g for 1 hr). High kinin-destroying activity has been reported in the soluble final supernatant fraction of homogenized rat liver (18), human granulocytes (9), rabbit brain (16, 19), and rat brain (20). Activity also has been found among particulate fractions of rabbit brain cell homogenates (19) and in a cell fraction of rat kidney homogenate that sedimented with the microsomal fraction (11).

Thus, this fibroblast cell line has both kinin-forming (1, 2) and kinin-destroying potential. These studies were undertaken following the observation that transplanted rodent tumors, with a mixed tumor cell-fibroblast cell population, have both an al-

kaline and acid kinin-forming enzyme and kininase activity (3, 21). Present data suggest that the fibroblast cell contributes to the overall kinin-enzyme system activity associated with the tumor tissue. While the role of fibroblasts in tumor growth and development awaits clarification, their proliferation at tumor transplant sites (22) and localization between the growing edges of necrotic areas in solid tumor transplants (23) would suggest some relationship with tumor growth mechanisms. Further study is required to establish whether these mechanisms are related to the vasoactive kinin protease system present in both the fibroblasts and tumor tissue. Reports of tumor inhibition by protease inhibitors (24, 25) provides indirect evidence for a possible role of proteases in malignancy.

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Experimental Infection of Human Foreskin Cultures with BK Virus, A Human Papovavirus¹ (39506)

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Introduction. BK virus (BKV), an SV40-related papovavirus, was first isolated by Gardner *et al.* (1) from the urine of a renal transplant recipient. The virus is a common infection of childhood (2, 3), but is not yet related to any illness. The virus probably persists in the individual after primary infection and is reactivated in times of immunological impairment, e.g., in transplant recipients and cancerous patients on immunosuppressive therapy (4-6) and in individuals with immune deficiency diseases (7). The virus transforms hamster cells in tissue culture (8) and produces tumors in neonatally inoculated hamsters (9).

BKV produces a lytic infection and grows to high titers in cells of human origin. Cells derived from human embryonic kidney (HEK) or brain and cells of the diploid fibroblast line WI38 are highly susceptible to lytic infection by the virus (10). We report here the results of BKV infection of early passage cell cultures derived from foreskins of newborn infants. In order to test the capacity of BKV to transform human cells, the lytic activity of the virus was decreased by irradiation of the virus and, in some instances, by the incorporation of BKV antiserum in the medium.

Materials and methods. Foreskin cultures. Foreskins from circumcisions were obtained from the Johns Hopkins Hospital, B-2 nursery. The donors were black or white infants, approximately 3 days of age. The tissue was minced with scissors and trypsinized to initiate cell cultures. Cells were grown in Falcon plastic 25-cm² tissue culture flasks in Eagle's minimum essential medium (Earle's salt base) supplemented with nonessential

amino acids, antibiotics, and 20% vated fetal calf serum and were maintained on the same medium but with 5% fetal calf serum. The cells were passed in culture until sufficient cells were obtained for experiments.

Virus. The prototype BKV strain by Gardner *et al.* (1) was used at passage levels (Table I). Virus was concentrated 10-fold by ultrafiltration at Amicon Stirred Cell using a PM-30 membrane. For irradiation, petri dishes containing virus were placed 15 cm away from a germicidal lamp (Sylvania G15T8, 40 W/cm (400 erg/sec/cm²) were used at the surface. The time of exposure to uv irradiation was varied.

Infection of cultures. Two experiments were done. In the first, third to fifth passage cultures derived from 19 foreskins were exposed to unirradiated virus or to virus irradiated for 8 min. In addition, cultures from 10 of these foreskins were exposed to virus irradiated, respectively, for 15 min. In the second experiment, seventh passage cultures from four of the foreskins were subjected to virus irradiated for 30 min. One group of virus-exposed cultures was subpassaged serially and maintained at passage levels and titers of the virus before treatment of cultures in the two experiments are indicated in Table I. Two-tenths liter (0.2 ml) of concentrated virus without irradiation, was adsorbed to three-fourths confluent cultures in 25-cm² flasks. The multiplicity of infection for the unirradiated virus was about TCID₅₀. As shown in Table I, irradiation for 8 min resulted in a 50-fold, and for 15 min in a greater than 10,000-fold, reduction in infectivity titers. The hemagglutinin activity of the unirradiated virus in both

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BLE 1. A SUMMARY OF THE EXPERIMENTAL PROTOCOL: VIRUS TITERS AND TREATMENT GROUPS.

BK virus					
	Cultures	Passage level	Titer of unirradiated virus ^a	Titer of irradiated virus ^a	Treatment of cultures
1	19 cultures at 3-5 passage levels	10 in vero, 1 in HEK	10 ^{7.9}	10 ^{7.2} (8 min of irradiation)	1 A, Inoculated with unirradiated virus 1 B, inoculated with irradiated virus 1 C, uninoculated
2	4 of above cultures at passage 7	9 in vero, 2 in WI38	10 ^{8.2}	10 ^{4.4} (30 min of irradiation)	2 A, inoculated with irradiated virus 2 B, inoculated with irradiated virus and maintained and passed with antiserum 2 C, uninoculated, maintained and passed with antiserum

^a50/ml in WI38 cell culture tubes, observed for 30 days.

s 1:640-1:1280 and was unchanged adiation.

ge of 2B cultures with BKV antisept for cultures in treatment group others exposed to irradiated or unirradiated virus were observed for BKV CPE subpassage and without addition of antiserum to the medium. The four in group 2B were subpassaged serially a week, at a 1:3 split ratio, for 9 weeks. Two flasks were prepared at each passage level, of which one was used for passage and the other held for observation. BKV hyperimmune rabbit serum hemagglutination-inhibition (HI) titer of 1:1280 was incorporated in the medium at a concentration of 0.1% for 2B.

beginning immediately following exposure to irradiated virus and then for a period of 1 week after each passage. Cultures at various passage levels held for observation were maintained without antiserum. Inoculated cultures, group 2C, were maintained in the same way as group 2B; they were subpassaged and maintained in medium containing BKV antiserum.

vation of cultures. Infected and uninoculated cultures were observed for BKV CPE for cell morphology. Representative cultures were fixed in absolute methanol and stained with Jenner Giemsa stain. Supernatant fluids were assayed for BKV antigen by immunofluorescence (IF) and hemagglutination each week in Experiment 1

and periodically in Experiment 2. Immunofluorescence (IF) tests for BKV viral and T antigens were done, as described (3), on cells grown on coverslips or LabTek slides (Miles Laboratories, Napierville, Virginia). The reference sera for viral antibodies were a BKV-immune rabbit serum and a human serum pool with high titers of BKV HI antibodies. Both of these sera were nonreactive in IF tests with BKV and SV40 T antigens. The reference T antibody serum was derived from hamsters bearing SV40 tumors. This serum reacted equally well with SV40 and BKV T antigens. For some of the cultures, concentrated supernatant fluids were examined, after negative staining, for virus particles by electron microscopy (EM) (6).

Results. Response of foreskin cultures to unirradiated and irradiated BKV. All inoculated cultures, whether they were infected with irradiated or unirradiated virus, eventually developed BKV CPE. The effect of increased time of irradiation was to progressively delay the onset of BKV CPE. The course of BKV infection in cultures of Experiment 1 is summarized in Table II. On Day 5 postinfection, in 14 foreskin cultures infected with unirradiated virus, an average of 24% of the cells exhibited BKV viral antigen by IF tests. The proportion of fluorescing cells was lower, averaging between 9.9 and 1.4% in the three groups of cultures which were infected with irradiated virus.

TABLE II. RESPONSE OF FORESKIN CULTURES TO UNIRRADIATED AND IRRADIATED BKV.

	Treatment of cultures			
	Unirradiated virus	Virus irradiated 8 min	Virus irradiated 10 min	Virus irradiated 15 min
1. Number of cultures examined	14-19	15-19	9-12	5-10
2. Percentage of immunofluorescent cells, 5 days postinoculation ^a	24 ± 3.4	9.9 ± 2.2	4.3 ± 0.8	1.4 ± 0.7
3. Time of onset of CPE, range (weeks)	1-2	2-6	4-6	5-9
4. Time of appearance of HA in supernate, range (weeks)	2	3-6	5-8	5-8
5. Proportion of cultures showing regrowth	13/19	7/19	4/12	1/10
6. Time of beginning of regrowth, range (weeks)	3-7	3-13	6-9	9

^a Mean percentage of immunofluorescent cells for all cultures in the group ± standard error of the mean.

BKV hemagglutinins in titers ranging from 1:16 to 1:4096 were detected in almost all cultures which exhibited BKV CPE, but were not detected in infected cultures prior to the development of CPE. All of the uninfected control cultures remained normal and were negative for viral hemagglutinins and IF.

All of the foreskin cultures were fibroblastic in appearance at the time of inoculation with the virus. BKV CPE was first manifest by the appearance of scattered large rounded cells which, with time, increased in numbers and led to a complete destruction of the cell sheet, leaving behind only matted debris attached to the culture flasks. The time between the first appearance of CPE and complete cell destruction varied from between 2 to 7 weeks for the different cultures. The supernatant fluids contained large amounts of BKV hemagglutinins during this period.

A significant proportion of the cultures which had previously undergone extensive BKV CPE showed regrowth of cells and remained persistently infected with BKV (Table II). The regrown cells were fibroblastic in some cultures, epithelioid in others, and mixed in still others. A culture flask often had some areas of fibroblastic and others of epithelioid cells. The cells grew very slowly, often taking weeks before there were enough for passage. Rounded cells and cells with vacuolated or granular cytoplasm were common. The supernatant fluids from these cultures were mostly negative for BKV hemagglutinins but had low levels of infectious BKV, as indicated by successful

isolation of the virus in inoculated WI38 cells. In IF tests, between 1 and 5% of the cells from regrown cultures stained for BKV viral and T antigens. Virions of papovavirus morphology were detected by EM examination, after negative staining, of concentrated supernatant fluids from some of these cultures. In observation periods ranging from 12 to 24 weeks after the initial infection, none of the regrown cultures became virus free or grew rapidly enough to permit frequent passage.

Regrowth and passage of two cultures (JV-1 and JV-2) following BKV CPE. In the second experiment, an attempt was made to further decrease the lytic activity of BKV. The time of virus irradiation was increased to 30 min and one group of cultures (2B) was maintained on antiserum and weekly serial passage.

BKV CPE developed in each of the four cultures of group 2A, which were maintained without antiserum and without passage. CPE was first seen 4-11 weeks postinoculation and was extensive within 1 to 2 weeks of onset. As in the first experiment, there was regrowth of cells with development of persistent infection in two of the four cultures.

In cultures of the treatment group 2B, BKV CPE was not seen as long as the cultures were maintained with BKV antiserum. At each successive passage level, all cultures which were held without further passage and without antiserum developed BKV CPE within 1-3 weeks of discontinuation of the antiserum. A total of 32 culture flasks representing cells from four foreskins at pas-

levels between 7 and 16 were observed. Following extensive BKV CPE in all flasks regrowth occurred in 18. The remaining cultures showed the characteristics described earlier of persistently infected cultures, viz. slow growth, cells with varying morphology, some cells showing BKV CPE and low levels of infectious BKV. Cultures 17 and 18 of the 18 flasks, representing 16th and 17th passage levels of one foreskin (74-7) grew rapidly after an initial period of slow growth and exhibited some of the properties of transformed cells. These cultures were designated JV-1 and JV-2, respectively, and were characterized with respect to presence of BKV-specific antigens, morphology, and susceptibility to BKV infection. All passages of the uninoculated cultures (2C) remained normal.

Characteristics of JV-1 and JV-2 cell cultures. The history of the JV-1 culture is given in Table III. Following inoculation of 10th passage culture with irradiated BKV, CPE was first seen after nine further passages, at 9 weeks postinoculation. Progression of CPE to 4+ and regrowth of the culture to a stage where it could be subcultured took 7 weeks. In IF tests, <0.01% of the cells in the first two passages postinoculation showed viral antigen, but this proportion increased to 25% by 9 weeks. During this period, a comparable proportion of cells stained for T antigen. In contrast, in regrown cultures, viral antigen-positive cells were absent or infrequent, while the proportion of T antigen-positive cells rapidly increased to >90% (Fig. 1) by the 19th passage and remained subsequently at that level. BKV hemagglutinins were absent in

periodic examinations of the supernatant fluids. The history of JV-2 was essentially similar to that of JV-1 except that viral CPE and regrowth occurred in the 10th passage of foreskin culture 74-7, instead of in the 16th passage as in JV-1.

JV-1 and JV-2 cultures were resistant to BKV infection (Table IV). Following inoculation of BKV at a multiplicity of greater than 100, these cultures did not develop viral CPE, were negative for BKV hemagglutinins in the supernatant fluids, and had only 0.01 to 2% of cells showing viral antigen. In contrast, control cells derived from the same parent foreskin were susceptible to BKV infection.

Cells of JV-1 and JV-2 cultures looked alike and were readily distinguished from the control cells (Figs. 2 and 3). The control cultures were fibroblastic with long, spindle-shaped cells, essentially uniform in size, arranged in whorls or bands. Multinucleated cells were uncommon and the cytoplasm was free of granules. The cells of JV-1 and JV-2 cultures were granular and pleomorphic. Stellate cells rather than spindle cells were predominant and multinucleated cells were commonly found. Some of the nuclei had an irregular outline. Cytoplasmic vacuoles were frequently seen. Rounding cells which appeared to detach from the glass were always present. The cytologic characteristics of these cells were similar to those described for SV40-transformed cells in "crisis" (11).

JV-1 and JV-2 cells were contact inhibited and did not reach high saturation densities. Their growth rate was not greater than that of the control cells as judged by

TABLE III. BKV CYTOPATHIC EFFECT AND REGROWTH (JV-1) IN CULTURES OF FORESKIN 74-7.

	Weeks of observation after exposure to irradiated BKV																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22-29
number of cultures in which	8	9	10	11	12	13	14	15	16								17	18	19	20	21	22-27
cytopathic effect	0	0	0	0	0	0	0	0	0	1+	progression to 4+ and regrowth						0	0	0	0	0	0
percentage of BKV viral antigen positive	<0.01	<0.01					1	2	25								<0.01	<0.01	0	0	0	0-≤0.01
percentage of BKV T antigen positive	<0.01	<0.01						5	25								25	50	90	80	90+	90+

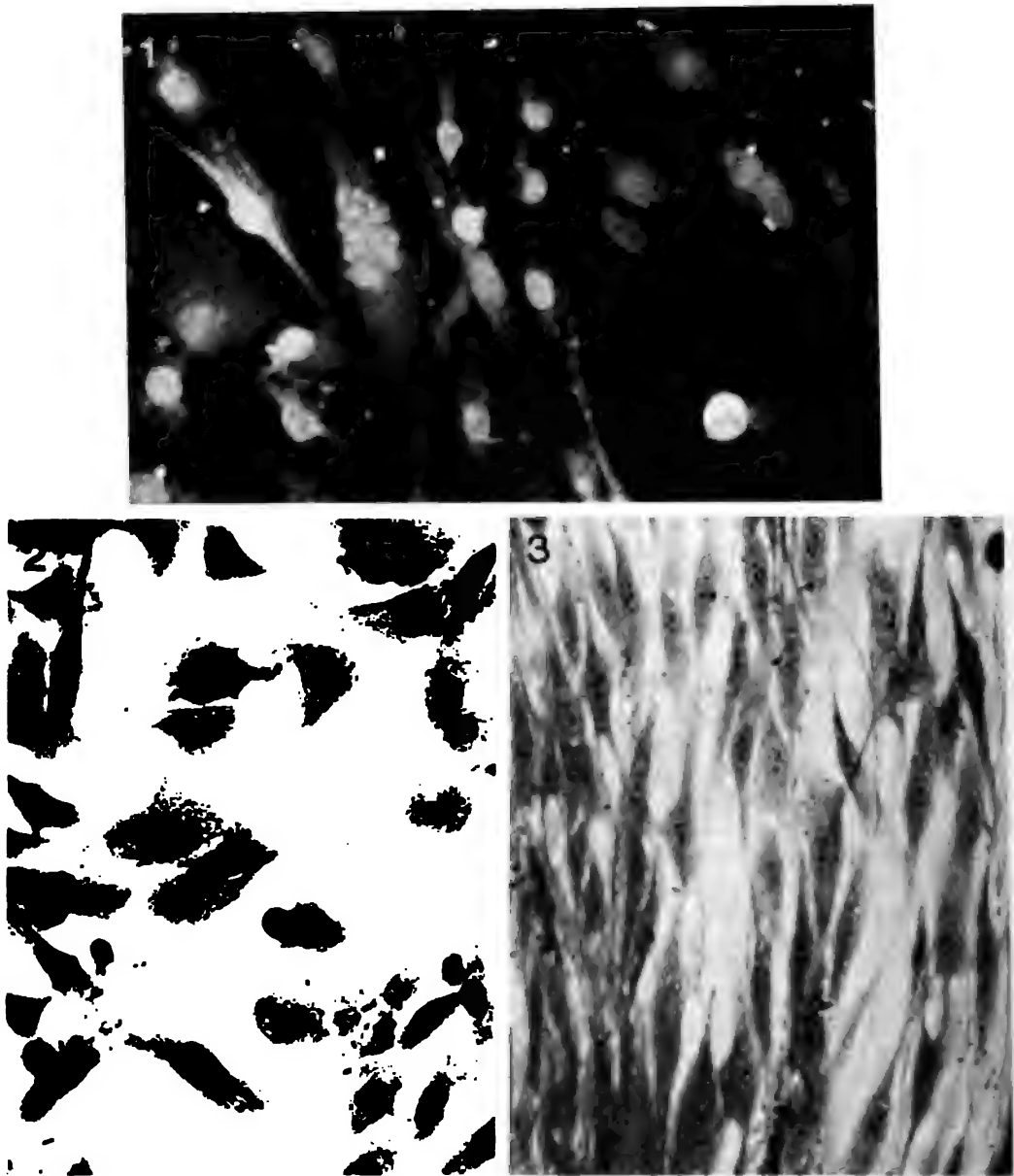


FIG. 1. Immunofluorescence test of JV-1 cells, passage 21, with SV40 T antibody showing the intranuclear T antigens. These cells were negative for BKV viral antigen. $\times 267$.

FIG. 2. JV-1 cells in a coverslip preparation showing lack of confluent growth and presence of numerous giant cells. The cells are stellate and nucleoli are prominent. Jenner-Giemsa stain. $\times 267$.

FIG. 3. Control human foreskin culture with a monolayer of uniform spindle cells containing regular oval nuclei. $\times 267$.

the time required to become confluent and by the number of cells recovered at various times after initiation of the cultures. JV-1 and JV-2 cells could not be maintained be-

yond the 30th passage since the primary culture.

Discussion. All of the 19 cultures derived from foreskins of newborn infants devel-

IV. RESPONSE OF JV-1, JV-2, AND NORMAL CELLS, ALL DERIVED FROM FORESKIN 74.7, TO BKV INFECTION.

Response	Days postinfection						
	3	8	12	16	20	24	28
Percentage with viral antigen	1	2					
cytopathic effect		0	0	0	0	0	0
hemagglutinin in supernate ^a			Neg ^b	Neg	Neg		
Percentage with viral antigen	0.01	0.01					
cytopathic effect		0	0	0	0	0	0
hemagglutinin in supernate ^a			Neg	Neg	Neg	Neg	
Percentage with viral antigen	20	50					
cytopathic effect		1+	2+	3-4+	4+	4+	4+
hemagglutinin in supernate ^a			32	256	1024		512

^aal of titer.^bmagglutinin titer of <1:10.

tic infection after inoculation of several investigators have successfully the lytic viral activity in per-cells by exposure of the virus to on; for instance, in the transformation of African green monkey kidney cells (12) and of hamster fibroblasts (13). In this study, infection of BKV and maintenance of cells with medium containing BKV was delayed but did not prevent viral cytopathic effect. Although regrowth occurred in about one-half of the cultures following extensive BKV CPE, these cultures were persistently infected and, as a rule, grew slowly. In only two instances, these cultures could be passed regularly; of these two cultures met some criteria of viral transformation. Their morphology was altered, and they had acquired resistance to infection with BKV. All cells possessed the intranuclear T antigen and the viral antigen was either not detectable in a very small proportion of cells. The cell cultures, however, did not exhibit the accelerated growth rate and saturation densities that are characteristic of transformed cell lines, and they could not be maintained beyond the 30th passage. Human cells transformed by SV40 virus also have a limited life span (14, 15).

Primary Cell Cultures. Cell cultures derived from foreskins of 19 newborn infants were exposed to irradiated or unirradiated BK virus or an SV40-related papovavirus infection, and were observed for viral

cytopathic effect (CPE) and for possible transformation. Four of the cultures were passed serially and maintained for a period of 9 weeks on medium containing BKV antiserum. All infected cultures developed viral CPE. Irradiation of virus delayed but did not prevent viral CPE. In nearly one-half of the cultures, some regrowth occurred following extensive virus-induced destruction of the cell sheets but the regrown cells, as a rule, remained infected with BKV and could not be passed. In two instances, regrown cells (JV-1 and JV-2) could be passed, and these had some of the characteristics of transformed cells. They were essentially free of BKV viral antigen but contained, in over 90% of the cells, the intranuclear T antigen. They were resistant to superinfection with BKV. In contrast to the uninfected control cells which were uniformly fibroblastic in appearance, cells in JV-1 and JV-2 cultures were pleomorphic with a predominance of stellate cells and multinucleated giant cells. However, JV-1 and JV-2 cultures were contact inhibited, did not reach high saturation densities, and could not be maintained beyond the 30th passage.

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port describes the effect of purified LPS on growth-related parameters in mouse embryo fibroblasts. In contrast to the results reported previously by *et al.* (3) on chicken cells, LPS has no effect by itself on cultured mouse cells. However, in the presence of calf serum, LPS exerts a potent synergistic effect on the stimulation of DNA synthesis in sta-

To assay for the stimulation of DNA synthesis, mouse embryo fibroblasts were

plated in D-MEM with 10% calf serum (2×10^5 cells per 35-mm dish to 2 ml of medium). The next day the medium was replaced with 2 ml of D-MEM containing 0.5% calf serum. Cells prepared in this manner entered a stationary resting phase in which little DNA synthesis occurred. Four or five days after the final medium change, cultures were used to assay for serum stimulation of DNA synthesis by replacing the medium with 2 ml of fresh medium with or without 10% serum and the various test substances. DNA synthesis was measured 18 hr after stimulation during the peak of "S" phase, by removing the medium and incubating the cultures for 1 hr in 1 ml of D-MEM containing $0.2 \mu\text{Ci}$ of [^3H]thymidine. Thymidine incorporation under these conditions was shown to be linear for periods greater than 2 hr and a linear relationship between incorporation and numbers of cells in S phase as determined by autoradiography was obtained (data not shown). At the end of this 1-hr pulse, the radioactive medium was removed, cultures were washed once with cold PBS and twice with cold 10% trichloroacetic acid (TCA) and fixed in ethanol:ether (3:1). Cultures were then air-dried, cells were dissolved in 0.2 N NaOH, and aliquots were added to 10 ml of aqueous scintillation fluid for determination of incorporated radioactivity. All points were done in duplicate, and duplicate samples did not vary by more than 10%.

Serum stimulation of cells prepared in this manner resulted in an approximate 10-fold increase in thymidine incorporation over the no serum controls (Fig. 2). Final cell density in the stationary cultures was about 8×10^4 cells/cm² and the monolayer had just reached confluence. In these stationary cultures less than 2% of the cells were shown to be in S phase at any one time as determined by autoradiography (data not shown) after a 1-hr pulse of [^3H]thymidine.

To determine the percentage of labeled nuclei, cells were labeled as described above with a 1-hr pulse of [^3H]thymidine. Cultures were washed twice with cold 10% TCA, fixed in ethanol:ether (3:1), and air-dried. Cultures were then coated with Kodak nuclear track emulsion NTB3 and exposed for 14 days at 4°. Autoradiographs

were developed and the percentage of labeled nuclei was determined using phase contrast microscopy. At least 1500 cells were counted per culture.

Calf serum and Dulbecco's modified Eagle's medium (catalog No. H-21 HG) were purchased from Grand Island Biological Co. Tritiated thymidine (40 Ci/mmol) was purchased from New England Nuclear. Bacterial lipopolysaccharides were purchased from Difco Laboratories. Lipopolysaccharide W from *Escherichia coli* 055:B5 was used unless indicated otherwise.

Results. The effects of lipopolysaccharides from *E. coli* on the growth rate of early passage mouse embryo fibroblasts were examined. As is evident from Fig. 1, the presence of LPS (20 $\mu\text{g}/\text{ml}$) produced a significant increase in cell number over those cells grown in the presence of 10% calf serum alone. This increase in growth rate was particularly evident during the first 48 hr and this margin of difference was maintained throughout the course of the experiment. Under these conditions the cells become confluent at a density of approximately 2.2×10^6 cells per dish. It is therefore evident that LPS is effective in enhancing the growth of both sparse and confluent cells.

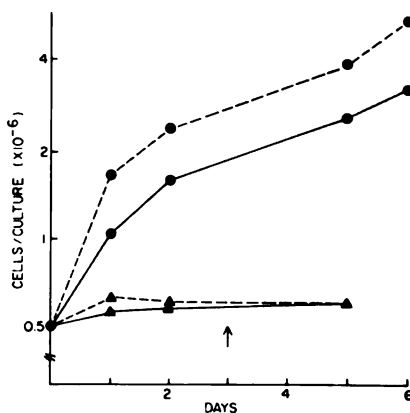
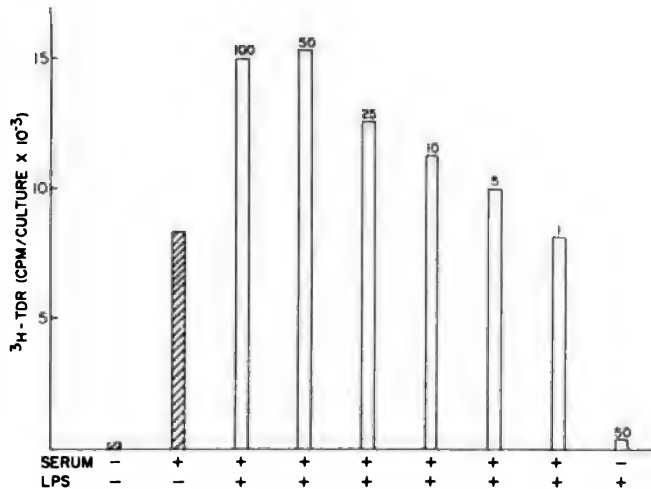


FIG. 1. Effect of LPS on the growth rate of mouse embryo fibroblasts. Cells (5×10^5) were seeded per 60-mm culture dish in 5 ml of DMEM containing 10% calf serum. After allowing the cells to attach, medium was removed and replaced with DMEM containing 0 (Δ) or 10% (●) serum with (—) or without (---) 20 $\mu\text{g}/\text{ml}$ of *E. coli* 055:B5 LPS W. Medium was changed on Day 3 as indicated by the arrow, and cell counts were performed at the times indicated.



2. Effect of LPS on the serum stimulation of DNA synthesis. Stationary cultures of mouse embryo ts were prepared by maintenance in low serum containing medium as outlined in Materials and Methods. fluid was changed to medium containing 0 or 10% serum with and without various concentrations of LPS. at the top of each bar indicate μg of LPS per culture (2 ml). Rates of DNA synthesis in the cultures were 18 hr later using a 1-hr pulse of [^3H]thymidine as described in Materials and Methods.

Is show that in the absence of calf cells do not grow and LPS is inactive LPS was not toxic to the cells and did ult in a loss of cells from the mono- Additional experiments using various trations of LPS did not yield results antly different from those shown in Concentrations less than 1 $\mu\text{g}/\text{ml}$ ithout effect and LPS was not active bsence of calf serum.

aw of this moderate, but significant, ement of the growth rate by LPS, it interest to investigate whether LPS also enhance the incorporation of midine by stationary cells stimulated esize DNA by changing the culture fresh medium containing 10% calf with or without various concentra- LPS (Fig. 2). Rates of DNA synthe e determined during the peak of S using a 1-hr pulse of [^3H]thymidine. a shown demonstrate that while LPS apparent stimulatory effect in itself, nces the serum stimulation of DNA is almost twofold. This maximum tory effect is seen using 50 μg of . Lower or higher concentrations of d a diminishing result. Thus, a syn- is seen when LPS is present in addi- calf serum resulting in an increase in

incorporation over that seen in the presence of either supplement alone. The results of the DNA synthesis experiment are therefore consistent with the early effects of LPS on the growth rate of mouse fibroblasts and seem to suggest that LPS is exerting a permissive effect on the cells and allowing more cells to enter the replicative cycle in a given period.

In order to verify this, autoradiographic experiments were performed to determine if the increase in [^3H]thymidine incorporation seen in Fig. 2 using LPS was due to an increase in the actual number of cells in S phase or due to increased rates of thymidine incorporation by the same number of cells. It is conceivable that the enhanced thymidine incorporation in the presence of LPS is due to an effect on thymidine transport allowing an increased uptake of the precursor by cells already stimulated by serum. However, the results shown in Table I clearly indicate that the twofold increase in [^3H]thymidine incorporation seen in the presence of LPS is due to a corresponding twofold increase in the number of cells actually engaged in DNA synthesis. It is therefore clear that the presence of LPS in addition to serum results in an increased efficiency of stimulation in that more cells are

TABLE I. EFFECT OF LPS ON THE PERCENTAGE OF LABELED NUCLEI.^a

Stimulating medium	[³ H]Thymidine incorporation (cpm/culture)	Percentage of labeled nuclei
No serum	210	3.3
No serum + LPS (50 µg/ml)	300	4.6
10% serum	1920	28.5
10% serum + LPS (50 µg/ml)	4200	61.0

^a Stationary cultures of mouse embryo fibroblasts were prepared as described in Materials and Methods. Each system consisted of four identical cultures that were stimulated, pulsed, and processed in exactly the same manner. Two cultures were hydrolyzed and used for determination of total incorporated radioactivity and two cultures were processed for autoradiography and determination of the percentage of labeled nuclei.

capable of responding to serum by synthesizing DNA if LPS is also present in the medium. Preliminary experiments (data not shown) verified that the results seen were not due to a shift in the time of S phase by LPS. The peak of DNA synthesis occurred at 18 hr after addition of serum with or without LPS.

To further characterize this cellular response to LPS, the effect of serum concentration was examined. The dose-response curves illustrated in Fig. 3 show that the synergistic effect of LPS is evident at all serum concentrations used and that the LPS has no effect in the absence of serum.

In order to determine whether the LPS is exerting its effect directly on the cell or on a component of the serum, the experiment described in Table II was performed. Cells were pretreated for 6 hr with LPS in serum-free medium prior to stimulation. Cell cultures were washed twice with PBS to remove all traces of LPS that were not cell associated. The data show that pretreatment with LPS in the absence of serum also results in a synergistic effect on the serum stimulation of DNA synthesis. While this experiment does not rule out the possibility that cell-bound LPS is reacting directly with serum growth factors at the cell surface, it strongly suggests that the mode of action of LPS is to cause an alteration in the cell membrane which results in a more efficient utilization of growth factors by the cell.

Discussion. The results reported indicate that LPS from the gram-negative bacterium *E. coli* is capable of exerting a striking synergistic effect on the DNA synthetic response of stationary early passage mouse embryo fibroblasts to serum. Data suggest that the LPS, which is ineffective by itself, is acting directly on the cell and is not altering a serum component. It appears that LPS exerts a permissive effect on the cells, enabling them to respond more efficiently to serum stimulation. Actually, twice as many cells respond to serum if LPS is also present in the medium or if the cells have been pretreated with LPS.

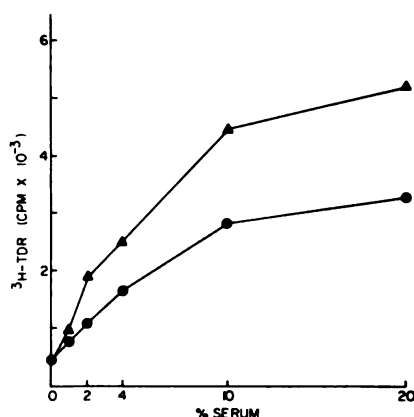


FIG. 3. Effect of serum concentration on the synergistic action of LPS. Stationary cultures of mouse embryo fibroblasts were prepared as outlined in Materials and Methods. Cells were stimulated by changing the medium to fresh DMEM containing 0 (●) or 25 µg/ml of LPS (▲) and various concentrations of serum. Rates of DNA synthesis were determined 18 hr later with a 1-hr pulse of [³H]thymidine.

TABLE II. EFFECT OF LPS PRETREATMENT.^a

Stimulating medium	LPS pre-treatment	[³ H]Thymidine incorporation (cpm/culture)
No serum	—	820
No serum	+	930
No serum + LPS	—	890
10% serum	—	4260
10% serum	+	8640
10% serum + LPS	—	9780

^a Stationary cultures were prepared as previously described. Some cultures were treated with 25 µg/ml of LPS for 6 hr in the absence of serum prior to stimulation.

otoxins (LPS) from gram negative bacteria are well-known mitogens of B lymphocytes. However, reports of the stimulation of nonlymphoid cells by LPS are very few. Vaheiri *et al.* (3) report that LPS of *Salmonella* sp. is a potent stimulator of DNA synthesis in chick embryo fibroblasts. Results shown in the present report are very different in that LPS has no activity but only acts synergistically with serum to promote the DNA synthetic response. This effect on confluent mouse embryo fibroblasts is only exerted at concentrations of LPS greater than 10 $\mu\text{g/ml}$. This effect is probably due to the cell type since chick fibroblasts are generally insensitive to growth-promoting agents in serum or insulin than are mouse fibroblasts. However, the experiments reported by Vaheiri *et al.* (3) were carried out in the presence of depleted serum and the results have been due to increased utilization of serum factors brought about by the addition of LPS.

Whether or not this interaction between serum and LPS is an operative biological process cannot be determined at this time. However, it is interesting to speculate that the presence of bacteria in wounds and infections may provide a natural environment which leads to synergistic stimulation of fibroblast proliferation by serum and bacterial endotoxins or other products. The mode of action for this permissive effect of LPS is not clear; however, an obvious site of interaction between serum factors and LPS would be the cell membrane. LPS are amphipathic molecules consisting of a highly polar polysaccharide and a nonpolar lipid portion (lipid A). It is known that LPS interacts with cell membranes and with artificial lipid bilayers. LPS may be acting in our system by altering or perturbing the membrane which might lead to an unblocking of receptor sites for growth factors in the membrane. The insertion of the lipid portion into the membrane may also affect membrane fluidity, a phenomenon which correlates with the mitogenic activity of concanavalin A (10, 11).

It is suggested that the synergistic response seen in this report provides a unique

opportunity to study serum-induced cell proliferation. Since the structure of LPS is generally known and techniques are available for alteration of that structure, effects of LPS on serum-induced DNA synthesis may provide a means of elucidating basic aspects of that interaction. Vaheiri *et al.* (3) have demonstrated that the carbohydrate portion of LPS is not important for the mitogenic effect of LPS on chick cells. This is in agreement with additional evidence that the biological activity of bacterial endotoxins resides in the lipid A moiety (12-14). Experiments are currently in progress to determine the mode of action of the LPS effect on DNA synthesis and to establish if this effect is common to all bacterial endotoxins and other similar bacterial products.

In addition, it should be noted that while LPS is a well-known B cell mitogen (4-7) and a T cell independent antigen, bacterial endotoxin has been demonstrated to have the ability to synergistically enhance the response of T cells to the mitogen Concanavalin A (15).

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A Procedure for Quantifying Susceptibility of Human Lymphocytes to Transformation by Epstein-Barr Viruses¹ (39508)

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in-Barr viruses (EBV) from diverse are capable of transforming human d cells with limited *in vitro* lifespan ublished cell lines (1-11). Surface- studies have shown that all EBV- ned human lymphoid cell lines pos- unoglobulins (Ig) (12-15), periph- phoid cells depleted of the Ig-bear- ulation are less susceptible or non- le to EBV transformation (16, 17), y surface Ig-bearing cells have EBV s (18). These findings suggest that y be specific for Ig-bearing or "B" ytes (14-17). Little is known, how- to whether all surface Ig-bearing ytes are equally susceptible and all earing lymphocytes are insuscepti- BV transformation. To answer this , one must devise a procedure to cell susceptibility to EBV transfor- We have successfully devised such y which determines the minimal of lymphoid cells that must be pres- culture in order for the culture to be ned by the EBV; results obtained s assay are reported here.

Materials and methods. Harvesting of peripheral leukocytes. In the early phase of this peripheral leukocytes were harvested entation in dextran (19), which a population of leukocytes contain- o 60% lymphocytes. Later, leuko- re collected by centrifugation of ve- od on a Ficoll cushion (20); leuko- tained by this method contained ymphocytes.

Preparation of Ig-bearing cells. Immunoab- columns were prepared by conju- bbit anti-human Ig or normal rabbit

immunoglobulins to CNBr-activated Sepha- rose 4B beads. The procedures described by other investigators were used with minor modifications (21-24). Peripheral lymphoid cells harvested by the Ficoll method were passed through the anti-human Ig or the control column. Cells not retained were ex- amined for surface markers and viability and then used in the study.

Surface markers. The proportion of cells that possess surface immunoglobulins was determined by staining with fluorescein- conjugated polyvalent antihuman Ig (25, 26). Under code, 100 to 300 cells were counted under the white light and the per- centage of cells showing three or more specks of granular fluorescence under uv illumination was enumerated. If negative, a total of at least 1000 cells was examined for fluorescent specks. The percentage of cells which formed rosettes spontaneously with sheep erythrocytes was determined as previ- ously described (27).

EBV preparations. One pool of a throat washing from a patient with infectious mononucleosis (7) and one pool of filtrate from the B95-8-transformed marmoset cell line (28) were used. The former contained $10^{1.5}$ and the latter contained $10^{4.5}$ trans- forming units per 0.2 ml when assayed on neonatal leukocytes (11).

Preparation of feeder cells. Human am- nion cells were harvested and grown as de- scribed (29). A cell suspension containing 50,000 cells/ml of medium was prepared and distributed in 1-ml portions to 18 × 150-mm culture tubes which were kept in the upright position for 1-3 days. Prior to experimentation, the medium together with some nonadherent cells was removed.

Nutrient medium. Eagle's basal medium containing the nonessential amino acids (30), penicillin G (50 µg/ml), streptomycin

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(50 μ g/ml), and 20% fetal calf serum was used.

Determination of the minimal number of lymphoid cells required for transformation (MNLRT). Quadruplicate cultures containing 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , and 10 viable leukocytes in 0.8 ml of medium were prepared in culture tubes (18×150 mm) filled with 5% CO_2 in air and closed with rubber stoppers. Two-tenths milliliter of EBV was added to each culture. Four cultures of 5×10^6 cells were kept as uninoculated controls to monitor for possible spontaneous transformation. The cultures were fed individually and observed for transformation as described (11). Transformation was signaled by the appearance of cell aggregates which increased in size and number together with increased metabolic activity 3 to 8 weeks after inoculation with the EBV. At the end of the eighth week, the MNLRT was calculated by the 50% endpoint method (31). No correction was made for the variation in the proportion of lymphoid cells in the leukocyte suspension. Assays in which uninfected cultures transformed spontaneously were considered not valid; of all the assays reported in this publication, there was only one assay whose control cultures transformed.

EBV antibody. EBV capsid antibody was tested by the indirect immunofluorescent test (33).

Results. Evaluation of the assay for the minimal number of lymphoid cells required for transformation (MNLRT). Results of 14 assays are summarized in Table I. Amnion feeder layers consistently reduced the MNLRT by about 1 log; this finding is consistent with the reports that feeder layers of macrophages or fibroblasts enhance transformation (16, 32). Increasing the dosage of EBV from $10^{1.5}$ to $10^{4.5}$ transforming units (TU) reduced the MNLRT by about 1 log. Using amnion feeder layers and $10^{1.5}$ TU, the MNLRT for the leukocyte of one donor tested on five occasions was 4.62 ± 0.19 log. With amnion feeder layers and $10^{4.5}$ TU, the MNLRT for the leukocytes of seven EBV-seronegative donors was 3.14 ± 0.27 log. These results indicated that the MNLRT assay yielded reasonably reproducible results and that the lowest MNLRT value was obtained by the use of amnion feeder layers and $10^{4.5}$ TU of EBV from the B95-8 marmoset cell line. These experimental conditions were used in all subsequent experiments.

Depletion of Ig-bearing cells on the MNLRT value. Results are summarized in

TABLE I. EFFECT OF FEEDER LAYERS, EBV DOSAGES, AND METHODS OF HARVESTING LEUKOCYTES ON THE MINIMAL NUMBER OF LYMPHOID CELLS REQUIRED FOR TRANSFORMATION (MNLRT).

Expt	Donors ^a	Harvesting of leuko- cytes	EBV ^b source and dose	MNLRT (in log)		
				With feeder	Without feeder	
1	IS	Dextran	TW	10 ^{1.5}	4.3	6.0
2	IS	Dextran	TW	10 ^{1.5}	4.7	5.5
3	IS	Dextran	TW	10 ^{1.5}	4.7	6.0
4	IS	Dextran	TW	10 ^{1.5}	4.8	NT
5	IS	Dextran	TW	10 ^{1.5}	4.8	NT
6	HC	Dextran	TW	10 ^{1.5}	3.8	4.5
7	HC	Dextran	B95-8	10 ^{1.5}	4	NT
			B95-8	10 ^{4.5}	2.8	NT
8	NB	Dextran	B95-8	10 ^{1.5}	4	NT
			B95-8	10 ^{4.5}	3	NT
9	DV	Dextran	B95-8	10 ^{4.5}	3.5	4.5
		Ficoll	B95-8	10 ^{4.5}	3	4
10	DV	Ficoll	B95-8	10 ^{4.5}	3.5	NT
11	TC	Ficoll	B95-8	10 ^{4.5}	3.0	NT
12	BO	Ficoll	B95-8	10 ^{4.5}	3.0	NT
13	GC	Ficoll	B95-8	10 ^{4.5}	3.0	NT
14	IS	Dextran	B95-8	10 ^{4.5}	3.5	NT

^a NB = newborn. All others were EBV-seronegative healthy persons (12-26 years old).

^b TW = throat washing; B95-8 = filtered spent medium from the transformed marmoset line (28). Dosages are in transforming units (11).

TABLE II. EFFECT OF DEPLETION OF SURFACE Ig-BEARING CELLS ON THE MNLRT.

Percentage of Ig-bearing and E-rosetting cells and MNLRT values		
Predepletion ^a	Depleted ^a	Sham depletion ^b
(44) (56), 3.0 ^c	(0.5) (77), 5.8	(5.5) (75), 2.8
(19) (53), 2.8	(<0.2) (72), 6.0	(6) (61), 2.8
(20) (53), 3.5	(<0.2) (69), >6.5	(10) (54), 4.0
(20) (45), 4.0	(1) (92), >6.5	(7) (75), 4.8

^a of EBV-seronegative donors was used in Expts. 1 to 3 and of an EBV-seropositive donor in Expt.

^berials and Methods. Sham depletion = cells not adherent to normal rabbit Ig-sepharose column. All suspensions contained >95% viable cells at the initiation of the assay.

^c), 3.0 = (44% surface Ig-bearing cells) (56% E-rosetting cells), and a MNLRT of 3.0 log.

The depletion of surface Ig-bearing cells from a lymphoid cell population decreased the MNLRT value by at least 2.5 log, indicating substantiates quantitatively the depletion of surface Ig-bearing cells and the susceptibility of lymphoid cells to EBV transformation (15-17).

MNLRT values for lymphoid cells from patients with chronic lymphocytic leukemia (CLL). The MNLRT values for the peripheral lymphocytes (over 90% surface Ig-bearing cells) of three CLL patients were 6.5, and >6.5 log. The assay for the transformation of the patient was not valid because of no transformation.

Conclusion. A procedure for determining the minimal number of leukocytes required for EBV transformation (MNLRT) by the EBV virus has been devised. Under the prescribed conditions, close MNLRT values were obtained. The average MNLRT value for peripheral leukocytes of seven EBV-seronegative healthy persons was 3.14 ± 0.27 log. It seems reasonable to consider a difference of 1 log in the MNLRT value as a

significant difference in susceptibility to EBV transformation; a higher value signifies greater susceptibility to EBV transformation. It is why a minimum of about 3 log of peripheral leukocytes must be present for EBV transformation.

It is not possible that only one of about 10^3 leukocytes is susceptible to EBV transformation; or, a minimum of about 10^3 transformed cells are necessary to create a detectable culture conditions for some transformed cells to grow into an established cell line. Whatever the explanation may be, it seems reasonable to consider the MNLRT value as a measure of the relative

susceptibility of a cell population to EBV transformation (defined as the ability of a cell population to grow persistently as a cell line). This statement is substantiated by our finding that depletion of the EBV-susceptible surface Ig-bearing cells from a lymphoid cell population increased the MNLRT values by at least 2.5 log.

Applying the assay to the peripheral lymphocytes of three patients with chronic lymphocytic leukemia (CLL), we found the CLL lymphocytes much less susceptible to EBV transformation. The MNLRT for the three CLL lymphoid cell populations were 6.5, >6.5, and >6.5 log in contrast to the value of 3.14 ± 0.27 log for the lymphoid cells from seven healthy persons. Since the CLL lymphocytes were predominately surface Ig-bearing cells, we conclude that there may be differences in susceptibility to EBV transformation among populations of surface Ig-bearing cells. The cellular basis for this low susceptibility of the CLL surface Ig-bearing cells to EBV transformation deserves further study.

Summary. An assay for determining the minimal number of leukocytes required for EBV transformation (MNLRT) by the EB virus has been devised. Close MNLRT values of 3.14 ± 0.27 log were obtained for the peripheral leukocytes of seven EBV-seronegative persons. Depletion of surface Ig-bearing cells increased the MNLRT value by at least 2.5 log. Applying this assay to the leukocytes of three chronic lymphocytic leukemia (CLL) patients we obtained the values of 6.5, >6.5, and >6.5 log.

Addendum: Since the submission of this manuscript the following relevant article has appeared:

Mizuno, F., Aya, T. and Osato, T. Brief Communication: Growth in semi-solid agar medium of human cord leukocytes freshly transformed by EB virus. *J. Nat. Cancer Inst.* **56**, 171 (1976).

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Curie	Ci	molar (concentration)	<i>M</i>
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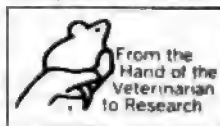


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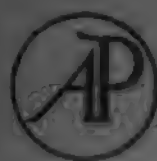
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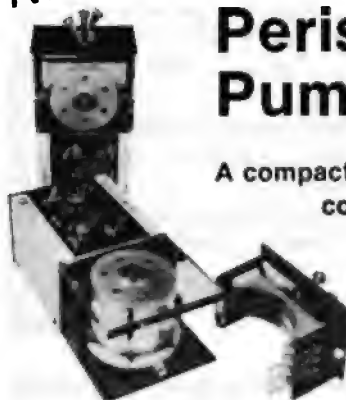
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Phase-Dependent Thermoregulatory Dysfunction in Pinealectomized Chickens (39509)

L. A. COGBURN, P. C. HARRISON, AND D. E. BROWN

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hormone-mediated effects of the pineal gland and endocrine system are well documented (1-3). Circadian and light-dark-entrained rhythms of melatonin (MT), norepinephrine (NA), and serotonin (5-HT) in the gland have been found in most vertebrates (4). Recently, the pineal gland has been implicated in thermoregulatory function of several vertebrates (5-9). Pineal glands of rats exposed to low ambient temperature underwent hypertrophy and hyperplasia (6). Pineal RNA and protein contents were reduced (7) and there was marked reduction of hydroxyindole-*O*-methyl transferase (HIOMT) activity in rats maintained at low ambient temperature (8). These findings support the hypothesis that the pineal gland is directly involved with integration of thermal stimuli.

Research for pineal involvement in the circadian and light-dark-entrained rhythms has been carried out in the rat (10) and house sparrow (5). Though pinealectomized rats exhibited no significant alteration of T_b rhythm, these animals had a lower temperature during the dark portion of their photoperiod, arrhythmia and a marked reduction in amplitude of circadian patterns of T_b following pinealectomy in sparrows housed in constant darkness. These findings indicate a correlation of the pineal gland or its secretory principles with physiological adaptations to changes in the thermal environment. Information is not now available on pineal involvement in thermoregulatory responses of birds to acute thermal challenge. The present experiment was designed to determine if pinealectomy would alter the thermoregulatory ability of chickens during acute exposure to a hot environment.

Materials and methods. Female day-old Columbian \times New Hampshire) from the University of Illinois Poultry Farm were housed in a battery brooder and maintained in a controlled-temperature chamber

on a 12L:12D photoperiod. Chicks were started at 35° with five subsequent decreases of 2.8° weekly. A standard broiler-starter ration and water were available *ad libitum*.

Pinealectomy (PX) and sham operations (PN) were performed at 8 days of age in a stereotaxic unit under Brevital Sodium (Eli Lilly and Co.) anesthesia. Birds were allowed to recover from anesthesia under a heating element before being returned to their cages. At 3 weeks of age the birds were assigned to six growing pens within two metal cages for an additional 6 weeks.

All birds were subjected to a single thermal challenge at 9 weeks of age in a completely randomized design with a factorial arrangement of three surgical treatments [PX, PN, and control (C)] and two times of day (diurnal and nocturnal). A single thermal challenge was administered to three birds simultaneously during the light phase and to three different birds during the dark phase of their photoperiod on each of 5 successive days. Each bird was challenged only once. Thermal challenges were started 4 hr after onset of daily light cycle (photophase) and 4 hr after onset of daily dark cycle (scotophase), respectively. This procedure resulted in thermal challenge to five birds from each surgical treatment during the photophase and five different birds from each surgical treatment during the scotophase, for a total of 30 individuals.

Prior to thermal challenge, each bird was fitted with a chest movement transducer and a thermistor rectal probe. Thermistor probes were calibrated daily against a mercury thermometer in a water bath. Rectal temperature (T_r) and respiratory rate (RR) were monitored continuously on three Gilson Unigraphs and a Gilson Polygraph, respectively. Five minutes after fitting of thermistor probes and chest movement transducers, recordings of T_r (degrees centigrade) and RR (breaths per minute) were

taken simultaneously from three birds at 23° as the zero-time measurement. The birds were then placed in a lighted Convicon (Controlled Environments, Pembina, N.D.) chamber maintained at 42°. After 60 min, the birds were removed from the chamber and held at 23° for an additional 20 min.

Dry-bulb recordings indicated chamber temperature was maintained at $42.2 \pm 0.2^\circ$ (mean \pm SEM) for 10 through 60 min of challenge at both times of the day. Dew-point temperature was not controlled, but recordings from all challenges show an average wet-bulb temperature of $35.4 \pm 0.2^\circ$ during heat challenge. Room temperature was $23.2 \pm 0.5^\circ$ during the recovery period. The T_r and RR were summarized at 10-min intervals throughout the thermal-challenge and recovery periods. The data were analyzed by the method of least squares, taking account of treatment, time of day, and challenge day. Least squares, rather than analysis of variance, was appropriate because challenge-day comparisons were not orthogonal to those for treatment and time of day.

Pineal uptake of trypan blue (11) was used to confirm total pinealectomy. Data from incompletely pinealectomized birds were not included in the analyses.

Results. The T_r of birds in each surgical treatment group are presented for diurnal (Table I) and nocturnal (Table II) heat episodes. There was no significant difference in prechallenge (0-min) T_r among PX, PN, and C groups, although prechallenge T_r was 0.3,

0.1, and 0.1° lower in respective groups at night. All groups had a comparable 7 to 10 and 20 min of exposure at both times of day.

Surgical treatment did not significantly affect T_r during the 60 min of diurnal thermal challenge; PX, PN, and C groups increased 2.2, 2.0, and 1.9° increases, respectively. At the end of the diurnal recovery period (80 min after heat exposure) T_r was 1.3, 0.5, and 0.1° above 0-min measurements for respective treatments. No differences between surgical treatments occurred at any time of diurnal challenge (Table I).

After 20 min of nocturnal heat, on the other hand, the hyperthermia of PX birds was greater ($P < 0.05$) than in PN birds (Table II). After 60 min of thermal challenge at night, the PX group increased a 3.3° rise ($P < 0.01$) in T_r above prechallenge temperature, while the PN and C groups rose only 2.2 and 2.3° , respectively (not significant). Similarly, the nocturnal PX group had a 1.4° higher T_r ($P < 0.01$) at the conclusion of the cooling period than the 80-min average for all groups, both nocturnal and diurnal (Table II).

Interactions between time of day and treatment recorded from each surgical treatment group are illustrated in Fig. 1. Each point represents the mean difference (nocturnal T_r minus diurnal T_r) of respective treatment groups at each measurement time. A significant nocturnal T_r is evident in all surgical

TABLE 1. EFFECT OF DIURNAL THERMAL CHALLENGE ON THERMOREGULATORY RESPONSE OF PINEALECTOMIZED PULLETS.^a

Time (min)	Body temperature (degrees)			Respiratory rate (br/min)		
	PX(5) ^b	PN(5)	C(5)	PX(5)	PN(5)	C(5)
0	42.5 ± 0.15^c	42.6 ± 0.09	42.6 ± 0.15	28.4 ± 1.8	44.8 ± 6.5	41.6 ± 1.2
10	43.0 ± 0.26	42.9 ± 0.08	42.8 ± 0.15	201.2 ± 34.7	153.6 ± 22.6	132.2 ± 10.1
20	43.6 ± 0.18	43.6 ± 0.14	43.4 ± 0.13	272.2 ± 15.7	238.4 ± 15.3	209.6 ± 10.1
30	44.0 ± 0.17	43.8 ± 0.16	43.7 ± 0.18	301.6 ± 7.9	284.8 ± 11.3	286.0 ± 10.1
40	44.2 ± 0.12	44.2 ± 0.19	44.0 ± 0.13	319.8 ± 20.4	270.8 ± 12.8	318.0 ± 10.1
50	44.4 ± 0.13	44.4 ± 0.22	44.2 ± 0.16	308.4 ± 10.6	285.6 ± 19.1	295.2 ± 10.1
60	44.7 ± 0.09	44.6 ± 0.31	44.5 ± 0.10	282.8 ± 18.2	274.8 ± 20.9	295.2 ± 10.1
70	44.0 ± 0.17	43.9 ± 0.29	43.6 ± 0.03	229.6 ± 38.9	214.4 ± 30.5	224.0 ± 10.1
80	43.8 ± 0.23	43.1 ± 0.13	42.7 ± 0.09	134.7 ± 32.4	110.5 ± 13.1	49.0^*

^a Diurnal thermal challenge (0 to 60 min at 42°) was given 4 hr after onset of light and was immediately followed by a recovery period from 70 to 80 min at 23°.

^b Surgical treatment group; number of birds in parentheses.

^c Mean \pm standard error.

* Significant difference ($P < 0.05$) between treatments.

TABLE II. EFFECT OF NOCTURNAL THERMAL CHALLENGE ON THERMOREGULATORY RESPONSE OF PINEALECTOMIZED PULLETS.^a

Body temperature (degrees)			Respiratory rate (br/min)		
PX(5)	PN(5)	C(5)	PX(5)	PN(5)	C(5)
42.2 ± 0.13 ^c	42.5 ± 0.21	42.5 ± 0.14	42.0 ± 3.9	42.0 ± 5.8	36.4 ± 4.0
42.8 ± 0.12	42.9 ± 0.20	42.9 ± 0.17	180.2 ± 23.1	172.4 ± 27.2	144.8 ± 28.0
43.6 ± 0.17	43.5 ± 0.12	43.4 ± 0.18	255.6 ± 11.2	244.4 ± 12.7	246.4 ± 20.7
44.4* ± 0.10	43.9 ± 0.12	43.7 ± 0.20	238.8 ± 15.6	269.2 ± 15.5	287.0 ± 18.0
44.9* ± 0.10	44.3 ± 0.07	44.2 ± 0.26	246.6 ± 18.3	280.4 ± 13.5	264.4 ± 20.2
45.3* ± 0.08	44.6 ± 0.07	44.5 ± 0.28	256.8 ± 16.5	274.4 ± 17.8	272.8 ± 26.9
45.5** ± 0.07	44.7 ± 0.08	44.8 ± 0.28	254.8 ± 19.6	299.6 ± 9.7	280.8 ± 28.5
45.1** ± 0.09	44.0 ± 0.20	43.5 ± 0.29	227.2 ± 14.4	260.2 ± 16.3	170.0* ± 24.0
44.3** ± 0.13	42.9 ± 0.14	42.6 ± 0.28	147.4 ± 21.4	146.8 ± 32.7	72.4* ± 18.5

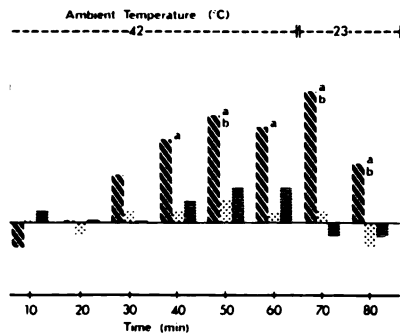
al thermal challenge (0 to 60 min at 42°) was given 4 hr after onset of darkness and was immediately a recovery period from 70 to 80 min at 23°.

l treatment group; number of birds in parentheses.

: standard error.

cant difference ($P < 0.05$) between treatments.

tes $P < 0.01$ level of significance.



Differences in body temperature of diurnal and nocturnal thermal challenges given to pinealectomized pullets. Each bar represents the nocturnal minus diurnal difference in a particular treatment group at each time. Significant differences between diurnal and nocturnal thermal challenges for a particular time are indicated by "a" ($P < 0.05$). Significant interactions between pinealectomy and time of day, i.e., diurnal versus nocturnal, are shown by "b" ($P < 0.05$).

initial recordings (0 min). The peripheral T_r of the nocturnal PX group, which began at 30 min, is well demonstrated.

Day-versus-night comparisons showed that the peripheral T_r was elevated after 40 and 50 min ($P < 0.01$) and all subsequent times ($P < 0.01$). Significant interactions between pinealectomy and time of day were found at 40 min of exposure ($P < 0.05$) and for the recovery period ($P < 0.01$). Although the peripheral T_r of the C groups had elevated nocturnal T_r during the recovery period from 40 to 60 min of heat

exposure, treatment by time-of-day comparisons revealed no differences. The only T_r difference ($P < 0.05$) between PN and C groups was at 70 min for both times of the day.

The RR during diurnal and nocturnal thermal challenges are presented in Tables I and II, respectively. No significant differences in RR between surgical treatments occurred during heat exposure. The highest diurnal RR occurred at 40 min for PX and C treatments (320 and 318 br/min, respectively) and at 50 min for the PN group (286 br/min). During the nocturnal episode, peak thermal tachypnea reached by the C group was 287 br/min at 30 min, while the PX and PN groups reached 257 and 300 br/min at 50 and 60 min, respectively. Recovery of RR was fastest in C birds, which showed a significantly lower ($P < 0.05$) RR at 70 min during the nocturnal challenge and at 80 min for both times of day.

Comparisons of RR between night and day challenges and interactions with surgical treatment groups are presented in Fig. 2. Day versus night comparison of RR showed differences at 30 min ($P < 0.05$) and 40 min ($P < 0.01$). A low RR (28 br/min) in the diurnal PX group at 0 min (Table I) produced a significant interaction ($P < 0.05$) between pinealectomy and time of day (Fig. 2). The nocturnal RR of PX birds was lower than the diurnal RR at all times of heat exposure. At 30 min of nocturnal heat,

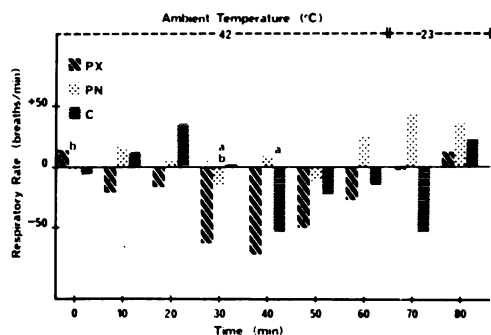


Fig. 2. Differences in respiratory rates for diurnal and nocturnal thermal challenges given to pinealectomized pullets. Each bar represents the nocturnal minus diurnal mean of a particular treatment group at each time interval. Significant differences between diurnal and nocturnal challenges for a particular time are indicated by "a" ($P < 0.05$). Significant interactions between pinealectomy and time of day, i.e., diurnal versus nocturnal, are shown by "b" ($P < 0.05$).

there was a consequent interaction with time of day ($P < 0.05$).

Least-squares analysis showed that the thermoregulatory response within treatment groups was independent of the day of the week and arrangement of treatments within a given challenge period.

Significant differences in body weights were not found between surgical treatment groups for either day or night heat episodes. The mean body weights (in grams) prior to nocturnal challenge were: (PX) 1233.6 ± 77.9 , (PN) 1113.6 ± 53.2 , and (C) 1160.2 ± 33.5 . Diurnal mean body weights (in grams) were 1211.2 ± 110.6 , 1217.4 ± 65.6 , and 1152.0 ± 53.3 for the PX, PN, and C groups, respectively.

Discussion. Several reports in the last decade referred to the association of the vertebrate pineal with maintenance of daily T_b patterns. Pineal and parapineal removal from lizards resulted in lowered body temperature and selection of higher ambient temperature (9, 12). Pinealectomy of house sparrows (*Passer domesticus*) caused a reduction in diurnal amplitude and disrupted the circadian pattern of body temperature (5). Similar to the results obtained in this experiment with chickens, nocturnal body temperature was consistently lower in pinealectomized rats (10). Our data indicate that a scotophase-dependent discontinuity

in the thermoregulatory response of pinealectomized domestic fowl occurs when the birds are exposed to an environment at 42° , for 60 min. Also it is noteworthy that at the conclusion of nocturnal recovery (80 min), the RR of the PX and PN were similar despite a 1.4° higher T_r in the PX group. This observation, along with a lower initial T_r , suggests that pinealectomy resulted in disruption of the thermoregulatory processes.

Unfortunately, the abrupt fall in RR of C birds during recovery for both times of day limits interpretation of differences between T_r and physiological mechanisms controlling evaporative cooling among treatment groups. The low RR encountered in C birds may be explained, as only the birds in this treatment had loss of plumage on their dorsal surface. This decreased insulation could have resulted in more rapid nonevaporative heat dissipation. Nonetheless, the loss of thermostasis observed in PX birds during nocturnal heat challenge suggests a pineal involvement in heat dissipation during scotophase.

Biogenic amines that are proposed to be involved in thermoregulation (13–17) exhibit metabolic rhythms in the pineal gland which are directly related to the daily light-dark cycle (4). These studies have involved several species of animals in a wide range of ambient temperature. Though conflicting data have been reported, the general consensus is that in mammals NA stimulates heat production whereas 5-HT facilitates heat loss (13), and it is proposed that a reversed role exists for monoaminergic participation in thermoregulation in avian species (18). The disruption of nocturnal heat-dissipating capacity of the pinealectomized chickens used in this study may reflect the absence of certain biogenic amines periodically produced or stored in the pineal that are important for efficient thermoregulation.

Changes in thermoregulatory responses following ablation of the pineal gland suggest the involvement of the pineal and its metabolites in thermostasis. At present, the locus of pineal participation in the control of T_b rhythms and in evaluation of thermal input from the environment remains unknown. However, it seems reasonable that

ion to transduction of photic input, al is also an intricate component in ogical adjustments to changes in am- perature.

ary. Pinealectomized female chick-) were given an acute thermal chal- 0 min at 42°) during two portions of L:12D photoperiod. Five birds from rgical treatment group, including perated (PN) and nonoperated con-), were exposed to heat 4 hr after f light (photophase) and a like num- hr after onset of darkness (scoto-

There were no differences in rectal ature (T_r) or respiratory rate (RR) treatment groups of the photophase allenge. Heat exposure during the ase caused a significantly greater (P)) hyperthermia in PX birds after 20 eat. At the conclusion of the noctur- lence, PX birds had a T_r 1.0° higher d and C birds. Time-of-day compari- RR showed a significant ($P < 0.05$) ion between pinealectomy and time- it 30 min of exposure; the RR of PX as lower at night.

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The Effects of Indanyloxyacetic Acid (MK 196) on Electrolyte Excretion in the Rat Kidney (39510)

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A substituted derivative of ethacrynic acid, 6,7-dichloro-2-methyl-1-oxo-phenyl-5-indanyloxyacetic acid (MK 196), has been shown in preliminary studies to be a potent uricosuric and diuretic agent in the rat and monkey (1, 2). Prior studies from this laboratory have localized the nephron site of inhibition of urate reabsorption to the proximal convoluted tubule. The reabsorption of sodium in the proximal tubule, however, is not affected by MK 196, and the sodium diuresis results from impaired reabsorption in the ascending limb of the loop of Henle (1). The current studies were undertaken to examine further the changes in electrolyte excretion induced by this agent and to gain additional inferential information on the nephron site of action.

Methods. Clearance studies. Male Sprague-Dawley rats weighing 200 to 400 g were used in all studies. Animals were anesthetized with Inactin (Promonta, Hamburg, Germany), 100 mg/kg body wt injected intraperitoneally, and prepared for clearance studies as previously described (3). A solution of isotonic saline containing [*methoxy*-³H]inulin (25 μ Ci/ml) was infused at a rate of 1.2 ml/hr for the duration of the experiment. MK 196 was dissolved in distilled water containing an equal concentration of sodium bicarbonate and infused in a dose of 50 mg/kg body wt/hr in a volume of 1.2 ml/hr. Control animals received the same solution without the drug. In the experimental animals, urinary losses of sodium and water were replaced with a volume of isotonic saline equal to the urine flow rate. Hematocrits were measured before and after the study to ensure the adequacy of volume replacement. After 60 to 90 min of equilibration, blood and urine samples were obtained.

Free water clearance and reabsorption studies. Studies were performed in a sepa-

rate group of animals that were lightly anesthetized with ether for placement of catheters, placed in restraining cages, and allowed to awaken. A solution of [*methoxy*-³H]inulin (25 μ Ci/ml) was infused at 1.2 ml/hr for the duration of the study. Free water reabsorption (T^cH_2O) studies were performed in three rats, deprived of food and water for 24 hr. Pitressin tannate in oil (0.5 U) was injected subcutaneously 1 hr prior to study. Two percent saline was infused at a rate of 0.20 ml/min. Free water clearance (C_{H_2O}) studies were performed in three animals deprived of food for 24 hr but allowed free access to 2% dextrose drinking solution. On the day of study a solution containing 1.67% of glucose, 1.3% ethanol, and 0.13% sodium chloride was administered by stomach tube in a volume equal to 3% of body weight. The water diuresis was sustained by the infusion of hypotonic saline (0.225%) at a rate of 0.20 ml/hr. In both groups of animals, blood and urine samples were obtained after a 60-min equilibration period, after which MK 196 was infused as a bolus (50 mg/kg body wt). Additional blood and urine samples were obtained 30 to 120 min after administration of the drug. The rate of saline infusion was not changed.

Radioactivity of blood and urine samples was determined in a modified Bray's solution in a Packard Tri-Carb liquid scintillation counter (Packard Instruments Co., Downers Grove, Ill.). Hematocrits were measured in microhematocrit tubes, Na⁺ and K⁺ by flame photometry, and uric acid by a specific uricase method utilizing a Beckman glucose analyzer (Beckman Instruments, Inc., Fullerton, Calif.). Ca²⁺ and Mg²⁺ were measured by atomic absorption spectrophotometry. PO₄ in serum and urine was determined in an AutoAnalyzer; pH was determined in an Instrumentation Laboratory blood gas analyzer (Instrumentation

tories, Watertown, Mass.). Osmolality determined by freezing point depression. An Advanced Instruments osmometer. Clearance and fractional excretions calculated from standard formulae. Control periods for each animal were advanced and the results expressed as the mean \pm SEM. Statistical significance was determined by the *t* test for unpaired data.

Results. Clearance studies (Table I). In the control animals, hematocrits measured prior to ($43.3 \pm 0.42\%$) and during ($43.3 \pm 0.23\%$) the infusion of the drug were not significantly different, indicating adequacy of volume replacement. The effect of the diuretic response was delayed, requiring 30 min. Compared to controls, continuous infusion of MK 196 resulted in significantly higher rates of urine flow, of excretion of sodium, and of urinary excretion of potassium. The glomerular filtration rate was not significantly lower in the control animals. The clearance and fractional excretion of urate were markedly reduced in the MK 196-treated animals as compared to controls. The clearances and fractional excretions of calcium and magnesium were also significantly higher in the treated animals. There was no significant change in phosphate clearance in response to MK 196.

Water clearance and reabsorption

To localize the nephron site of iminodisodium reabsorption, T^3H_2O and studies were performed and the results summarized in Table II. In response to a

bolus infusion of MK 196, fractional free water reabsorption as a function of fractional osmolar clearance and fractional free water clearance as a function of fractional volume flow were decreased below values obtained during the control periods.

Discussion. MK 196, a substituted derivative of ethacrynic acid, has previously been reported to be a potent diuretic and uricosuric agent in the rat (1). The current studies confirm our previous findings on the renal effects of MK 196 on sodium and urate excretion and present additional information on its effects on the excretion of calcium, magnesium, phosphate, C_{H_2O} , and T^3H_2O . Following the intravenous infusion

TABLE II. THE EFFECT OF MK 196 ON C_{H_2O} AND T^3H_2O .^a

C_{H_2O} ($n=3$)			
Fractional volume excretion (%)		Fractional C_{H_2O} (%)	
C	E	C	E
6.63 ± 0.42	9.71 ± 1.58	4.60 ± 0.33	1.36 ± 0.31
P, NS		T^3H_2O ($n=3$) $P < 0.001$	
Fractional osmolar clearance (%)		Fractional T^3H_2O (%)	
C	E	C	E
14.52 ± 0.90	15.76 ± 0.78	15.76 ± 0.78	3.4 ± 0.30
P, NS		$P < 0.001$	

^a Numbers represent mean \pm SEM; NS = not significant; C = control; E = experimental; n = number of animals studied.

TABLE I. RENAL EFFECTS OF MK 196.^a

	Control (6)	Experimental (7)	<i>P</i>
U/min/g KW)	1149.0 \pm 118.6	906.0 \pm 54.6	NS
(μ l/min/g KW)	8.9 \pm 1.4	119.0 \pm 28.8	<0.005
equiv/min)	0.40 \pm 0.039	26.1 \pm 2.2	<0.001
equiv/min)	3.3 \pm 0.6	6.2 \pm 0.7	<0.01
/min/g KW)	37.7 \pm 5.9	137.0 \pm 38.0	<0.025
l/min/g KW)	131.8 \pm 13.7	464.0 \pm 83.5	<0.005
(%)	11.4 \pm 0.3	56.4 \pm 10.7	<0.001
(μ l/min/g KW)	4.7 \pm 0.6	21.0 \pm 0.5	<0.01
(%)	0.4 \pm 0.05	2.0 \pm 0.5	<0.005
min/g KW)	35.0 \pm 9.0	130.0 \pm 19.0	<0.001
)	3.0 \pm 0.8	21.8 \pm 5.0	<0.01
(μ l/min/g KW)	163.0 \pm 40.0	179.1 \pm 100.0	NS
(%)	17.0 \pm 5.9	23.0 \pm 1.6	NS
	6.7 \pm 0.40	6.5 \pm 0.16	NS

Numbers represent mean \pm SEM. Numbers in parentheses indicate the number of animals studied.

of MK 196, there is a delayed onset of response which averaged 30 to 45 min. The reason for this delayed response is unknown but may indicate a requirement for further metabolism of the drug. MK 196 administration resulted in significantly higher rates of sodium excretion in the urine and a decrease in both C_{H_2O} and T^*H_2O . It is evident from these results that the major site of action of this agent is in the loop of Henle. These findings are consistent with the results of micropuncture studies which have indicated that fractional reabsorption in the proximal convoluted tubule was not significantly altered by MK 196 (1). C_{H_2O} and T^*H_2O studies, performed during the sustained infusion of the drug, revealed results qualitatively similar to those of the present study utilizing only a single bolus infusion of the drug (1). It would appear, then, that MK 196 has not only a delayed onset of action but also a relatively long duration of action.

In the present studies, the fractional excretion of urate was increased from 11 to 56%. This action on urate is localized to the proximal tubule where MK 196 has been demonstrated to inhibit both the reabsorption and secretion of urate, the net effect being an increase in urate excretion (1). The demonstration of impaired proximal urate reabsorption and normal sodium reabsorption in the proximal convoluted tubule suggests that the reabsorption of these two components of the glomerular filtrate is not intimately linked at this nephron site and that the effects of MK 196 on urate transport are specific.

The renal handling of other electrolytes may then be used to gain further inferential information on the nephron sites of action of MK 196. In the nonparathyroidectomized animal, the bulk of phosphate reabsorption occurs in the proximal tubule (4, 5). In the current studies, neither the clearance nor fractional excretion of phosphate was altered by MK 196. Although some degree of inhibition of phosphate reabsorption cannot definitely be excluded, it seems likely that MK 196 has little effect on phosphate transport, and these results further support the conclusion that the effects of

MK 196 on urate transport are specific and not the result of a generalized inhibition of proximal tubular function. Recent evidence has suggested that, in the rat, calcium is reabsorbed both in the proximal tubule and at distal nephron sites (6). Magnesium reabsorption, on the other hand, appears to occur mostly at sites beyond the proximal convoluted tubule (6, 7). The increase in calcium and magnesium excretion, in the absence of changes in phosphate excretion, are consistent with the conclusion that the increases in calcium and magnesium excretion are the result of inhibition of the reabsorption of these ions at a distal site.

Taken together, the results of these studies indicate that MK 196 is a potent loop-acting diuretic resulting in significant increases in the urinary excretion of sodium, calcium, magnesium, and water. In addition, MK 196 induces a marked increase in the urinary excretion of uric acid and thus might be a useful agent in some clinical settings.

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arent infections are a major source for maintenance and spread of the visible viral diseases of man. This is true for those diseases in which are the only reservoir of infection, for poliomyelitis, and probably for as well. It has been known for years that viral hepatitis may be sub- (1, 2) but the lack of means for finding either virus or antibody pre- vention precise assay of the incidence of infection.

development in recent years of methods for detecting hepatitis B antigen and antibody provided reliable means for diagnosing inapparent hepatitis B infections. By serum neutralization, immune precipitation (IA), and complement-fixation at procedures for detecting antibody to hepatitis A virus were described by Poppe (3-7) and these, together with the electron microscopy technique of Frazer *et al.* (8), opened the way to studying apparent infection in hepatitis A as heretofore, such infections could be detected only from the presence of abnormality of liver function that might be detected in fact, by any of a number of different methods.

This report presents the serological test for hepatitis A and B in a number of

patients were identified in the course of longitudinal epidemiologic studies of hepatitis carried out in an endemic hepatitis area in Costa Rica (9).

Materials and methods. Twenty-five persons with subclinical hepatitis, from whom appropriate serum samples were available, were selected for study. The presumptive diagnosis of subclinical hepatitis was based on serum glutamic pyruvic transaminase (SGPT) elevations found during weekly or bi-weekly monitoring of family contacts of clinically overt cases of viral hepatitis. In most individuals, the diagnosis was supported by measurements for serum bilirubin, for immunoglobulin M (IgM), and for hepatitis (HB_sAg) antigenemia.

Tests for hepatitis A antibody were performed by the IA test on the first serum specimen that showed an elevated transaminase level (acute specimen) and on a serum sample taken from the same individual about 90 days later (convalescent specimen). In some cases, serum samples taken earlier than the acute specimen were available for study. Hepatitis A antigen was prepared from the livers of infected *Saguinus mystax* marmosets. The IA test was carried out as described earlier (6, 10). The IA tests were performed at West Point, Pennsylvania. The sera were submitted under con-

the method of Reitman and Frankel (13) and for IgM by the method of Mancini *et al.* (14). Values exceeding 75 U/ml and 200 mg/100 ml (200 international units), respectively, were considered to be abnormal and of diagnostic significance.

Results. The findings in the tests of the sera from the 25 cases of clinically inapparent hepatitis are shown in Table I. All persons except the four subjects who showed hepatitis B antigenemia had been diagnosed tentatively as hepatitis A cases. The IA tests showed that 18 of the cases developed hepatitis A antibody. In all but three of these cases (No. 11, 18, and 21), the first sample of serum tested was free of detectable hepatitis A antibody. In these three individuals with low initial IA titers, the acute serum specimens had been taken 2-3 weeks after onset of serum enzyme elevation. All 18 persons developed high titers of hepatitis A antibody during convalescence.

Four of the 25 cases were diagnosed as hepatitis B (No. 1, 2, 4, and 5) because

HB_sAg was detected in their acute phase bloods; this was transient and disappeared within 3 weeks. All had resided in households where clinical hepatitis B had occurred. All had demonstrable hepatitis A antibody initially and there was no change in titer during convalescence.

Three of the 25 cases (No. 3, 6, and 24) had hepatitis B antibody at 1:8 to 1:32 titer in their acute phase sera, and there was no change in titer during convalescence. Two of these three persons (No. 6 and 24) had hepatitis A antibody in their acute sera and this did not increase in convalescence. Case 3 was without hepatitis A antibody initially and this person did not develop such antibody until 1 year later, at which time he was convalescent from a subsequent clinically apparent hepatitis A infection. The evidence indicates that these three persons had neither hepatitis A nor B and suggests that they may have suffered from hepatitis of different etiology (10, 15).

Serum samples were available over a fol-

TABLE I. DEVELOPMENT OF ANTIBODIES AGAINST HEPATITIS A VIRUS IN SUBCLINICAL CASES.

Case number	Sex	Age	SGPT (highest value)	IgM (mg/100 ml) acute	Immune adherence antibody titer			Hepatitis B antigen
					Pre onset ^a	Acute ^b	Convalescent ^c	
8	M	1	1660	>400	ND ^d	<5	≥6400	Negative
12	F	2	1260	>400	ND	<5	≥6400	Negative
9	M	2	920	>400	ND	<5	≥6400	Negative
13	F	1	830	210	ND	<5	≥10,240	Negative
23	M	2	830	290	ND	<5	≥6400	Negative
10	F	2	750	>400	ND	<5	≥10,240	Negative
15	M	6	680	>400	ND	<5	≥6400	Negative
7	F	2	415	ND	ND	<5	≥6400	Negative
16	F	2	415	180	<5	<5	≥6400	Negative
19	F	2	375	340	<5	<5	3200	Negative
14	F	5	345	>400	<5	<5	5120	Negative
17	M	2	310	>400	<5	<5	≥6400	Negative
22	F	7	230	150	ND	<5	3200	Negative
20	M	6	126	>400	<5	<5	3200	Negative
25	F	1	114	ND	ND	<5	≥6400	Negative
21	F	3	280	>400	<5	200	≥6400	Negative
11	F	6	310	ND	ND	800	≥6400	Negative
18	F	3	1030	>400	50	800	≥6400	Negative
2	F	6	100	110	ND	100	100	Positive
4	M	39	95	165	ND	400	400	Positive
1	M	7	120	ND	ND	≥6400	≥6400	Positive
5	M	7	1260	210	ND	≥6400	≥6400	Positive
3	M	2	134	>400	ND	<5	<5	Anti-HB _s Ag positive
24	M	54	230	70	ND	100	100	Anti-HB _s Ag positive
6	F	5	345	95	ND	≥10,240	≥10,240	Anti-HB _s Ag positive

^a Average: 14 days before onset.

^b At time of first detection of SGPT elevation.

^c Average: 90 days after onset.

^d ND = not done.

period of 2-7 years from the persons in the I who had developed hepatitis A only. In all cases, the antibody titers remained undiminished from the levels as in Table I.

As of interest that the IgM values in these A cases were most often 200 or more (13/15 cases) and the values in hepatitis B were less than 200 in two of three. This differentiating characteristic for levels in hepatitis A and B cases has been noted by Giles and Krugman (16). *Discussion.* The sensitivity and specificity of the IA test for hepatitis A antibody have been demonstrated by our group (4, 6, 7, 8) and have been confirmed in tests by Hagan *et al.* (17). In earlier studies (4, 6, 8) had seen evidence, in a limited number of cases, for antibody development in subclinical hepatitis A infection. Also, Hagan *et al.* (18) have shown antibody development, by immune electron microscopy, in similar subclinical hepatitis A cases characterized by serum enzyme elevations. These present findings confirm the reliability of the IA test for serologic diagnosis of hepatitis A, even in subclinical disease. Importantly, persons with subclinical hepatitis A, who rose with clinically apparent illness, had a high level of antibody against hepatitis A during convalescence from the disease and this antibody persists for at least 7 years. Such antibody likely equates with immunity to reinfection in hepatitis A, as Hagan *et al.* (19) have shown that resistance to reinfection follows both clinical and subclinical disease.

The lack of hepatitis A antibody response in four cases of hepatitis B was in accord with expectation and served to emphasize the specificity of the test for hepatitis A only. The three cases of inapparent infection for which no serologic diagnosis could be made were probably neither hepatitis A nor B and may, indeed, have been cases of the all hypothetical "hepatitis C" (10, 15). The newly developed IA test for hepatitis A, used in conjunction with the available tests for hepatitis B antigen and antibody, has opened the door to agent-specific serologic and epidemiologic investigations of these two important diseases of man. The

ability to detect both hepatitis A and B now presents the opportunity for a more rational approach to studies aimed at propagating these agents in the laboratory.

Summary. A newly developed immune adherence (IA) test for hepatitis A antibody was applied to etiologic investigations of subclinical hepatitis cases diagnosed by serum enzyme elevations. Among 25 cases of subclinical infection occurring in Costa Rica, 18 seroconverted and were diagnosed as hepatitis A, four had HB_sAg and were diagnosed as hepatitis B, and three were neither hepatitis A nor B. The development of hepatitis A antibody in such cases equates with protection against the disease and confirms that immunity may result from clinically inapparent infection. Hepatitis A antibody in these subclinical cases was shown to persist for at least 7 years, the longest time period investigated. The antibody response in subclinical hepatitis A was comparable to that previously noted in clinical cases of the disease.

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Arterial Responses to Three Prostaglandin Endoperoxide Analogs in the Dog (39512)¹

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Dogs, the bisenoic prostaglandin synthetase, arachidonic acid (AA), in an intravenous dose of 300 $\mu\text{g/kg}$, has a depressor effect of short duration blocked by PG synthetase inhibitors. Tachyphylaxis does not develop. AA is not directly a vasoconstrictor, however, it constricts canine pulmonary circulation in the isolated canine hindlimb (3). The effect of AA on contractile force in the dog is affected by AA, although a reflex inotropic effect may be observed.

There is a noticeable delay in the time of onset of hypotension and the inhibitory response by aspirin suggested that cardiovascular effects were due to AA metabolism. However, these are not those observed with $\text{PGF}_{2\alpha}$, which has a direct positive inotropic and blood pressure effect. We propose, therefore, that the cardiovascular actions were due to endoperoxide intermediates formed transiently in the bioconversion of PG endoperoxides to PGE_2 and $\text{PGF}_{2\alpha}$.

Analogs of PG endoperoxides have been available in limited quantities for testing (5, 6). We have examined the effects of these newly synthesized analogs on dog circulation, in a manner similar to that in which the properties of PGE_2 and $\text{PGF}_{2\alpha}$ have been characterized, to gain insight into the pharmacological properties of the intermediate derived from AA.

and methods. Mongrel dogs of various ages were anesthetized with sodium pentobarbital (30 mg/kg) and maintained on intermittent positive pressure respiration with a Harvard respirator. A left thoracotomy was performed. In three dogs, a Wal-

ton-Brodie strain gauge arch was sutured to the right ventricular wall for measurement of myocardial contractile force. In three additional dogs, a small catheter was inserted into a branch of the left pulmonary artery for direct recording of pulmonary arterial pressure. In each dog, a femoral artery and vein were catheterized for direct measurement of systemic arterial pressure and for administration of test substances directly into the inferior vena cava.

Two cyclic ether endoperoxide analogues were generously provided by Dr. G. L. Bundy of the Upjohn Company. These are: (15*S*)-hydroxy-9 α ,11 α -(epoxymethano)-prosta-5*Z*,13*E*-dienoic acid, referred to as U44069, and (15*S*)-hydroxy-11 α ,9 α -(epoxymethano)-prosta-5*Z*,13*E*-dienoic acid, referred to as U46619. Dr. E. J. Corey of Harvard University kindly provided 0.3 mg of an *azo*-endoperoxide analog: (15*S*)-hydroxy-9 α ,11 α -(*azo*)-prosta-5*Z*,13*E*-dienoic acid. (We have referred to this compound in this paper as *azo*.)

The three endoperoxide analogs were dissolved in ethanol to provide 1 mg/ml solutions. These were diluted in saline to 20 $\mu\text{g/ml}$. Hexamethonium chloride (Schwarz/Mann) was prepared in aqueous solution in a concentration of 50 mg/ml. Indomethacin (Merck) was prepared in dilute sodium carbonate as a 2 mg/ml solution for use intravenously.

Following observation of the initial cardiovascular response to each of the endoperoxides, ganglionic blockade was induced with hexamethonium (2 mg/kg) in three dogs and the test doses were repeated. Similarly, after initial observations, two animals received indomethacin to block PG synthetase (2 mg/kg), and the endoperoxides in the test doses were repeated. Fewer test doses of the *azo* compound were given owing to the limited amount available.

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Results. Systemic arterial pressure. Each of the three endoperoxide analogs is predominantly pressor (Table 1). One injection of the *azo* compound at a dose of 0.5 $\mu\text{g}/\text{kg}$ increased systolic and diastolic arterial pressure by 16 and 20%, respectively. Administration of 2.5 $\mu\text{g}/\text{kg}$ increased the systolic pressure in four dogs by 25% ($\text{SE} \pm 3.5$) and the diastolic pressure by 23% ($\text{SE} \pm 1.0$).

In six dogs, 1.25 $\mu\text{g}/\text{kg}$ of U46619 caused a mean systolic rise of 13.7% ($\text{SE} \pm 5.5$) and a mean diastolic rise of 17.5% ($\text{SE} \pm 4.7$). A larger dose of 2.5 $\mu\text{g}/\text{kg}$ caused 40 and 38% increases in systolic and diastolic pressures, respectively, in one dog. In seven dogs, 1.25 $\mu\text{g}/\text{kg}$ of U44069 caused a mean systolic rise of 17.0% ($\text{SE} \pm 4.3$) and a mean diastolic rise of 17.3% ($\text{SE} \pm 2.8$). A single dose of 2.5 $\mu\text{g}/\text{kg}$ caused 47 and 49% increases in systolic and diastolic pressures, respectively. The duration of these pressor responses ranged from 5 to 10 min. The typical manner of blood pressure rise, following a transient fall of several seconds duration, for each of these three endoperoxide analogs is shown in Figs. 1 and 2.

Pulmonary arterial (PA) pressure. Each one of the endoperoxide analogs is markedly vasoconstrictor in the pulmonary circulation. Immediate elevations of PA pressure following intravenous administration were as follows: Injection of *azo* (2.5 $\mu\text{g}/\text{kg}$) caused a 50% rise in systolic and 140% rise in diastolic pressure in the PA. Three injections of U46619 (1.25 $\mu\text{g}/\text{kg}$ intravenously) caused a mean PA systolic pressure rise of 55% ($\text{SE} \pm 7.7$) and a diastolic rise of 158% ($\text{SE} \pm 78$). Three injections of U44069 (1.25 $\mu\text{g}/\text{kg}$) caused a mean PA systolic

pressure rise of 83% ($\text{SE} \pm 36$) and diastolic rise of 132% ($\text{SE} \pm 47$). The configuration of the PA pressure rise is shown in Fig. 2. **Myocardial contractile force (MC).**

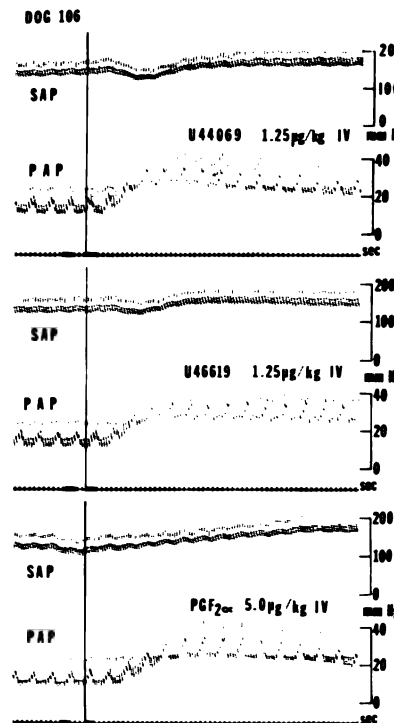


FIG. 1. Simultaneous recordings of systemic arterial pressure (SAP) and pulmonary arterial pressure (PAP) in a dog receiving the doses of U44069, U46619, and $\text{PGF}_{2\alpha}$ shown. In each case the vertical line represents the instant of introduction of the substance into the inferior vena cava. The transient fall in SAP in response to the endoperoxide analogs is interpreted as due to momentarily decreased return to the left heart because of rapid and intense pulmonary constriction. This is sometimes also observed with $\text{PGF}_{2\alpha}$.

TABLE 1. SYSTEMIC ARTERIAL PRESSURE (SAP) RESPONSES TO ENDOPEROXIDES

Compound (dose (n))	Initial blood pressure (systolic/diastolic, mm Hg)	Highest BP response (systolic/ diastolic, mm Hg)	Percentage of change systolic/diastolic, \pm SE
Azo (Corey) 2.5 $\mu\text{g}/\text{kg}$ (4)	159 \pm 6.6/122 \pm 2.5	199 \pm 10.7/150 \pm 3.5	25.2 \pm 3.5/22.8 \pm 3.5
U46619 1.25 $\mu\text{g}/\text{kg}$ (6)	168 \pm 4.2/126 \pm 4.0	191 \pm 13.1/148 \pm 10.4	13.7 \pm 5.5/17.5 \pm 5.5
U44069 1.25 $\mu\text{g}/\text{kg}$ (7)	165 \pm 8.4/127 \pm 6.4	193 \pm 10.0/149 \pm 8.4	17.0 \pm 4.3/17.3 \pm 4.3
$\text{PGF}_{2\alpha}$ 5 $\mu\text{g}/\text{kg}$ (7)	172 \pm 6.1/131 \pm 4.6	199 \pm 10.5/154 \pm 8.4	15.7 \pm 4.7/17.6 \pm 4.7

endoperoxide analogs is directly positiveotropic (Fig. 2). In three dogs, *azo* ($\mu\text{g/kg}$) caused a mean 13.5% increase in MC (SE ± 2.9). Following ganglionic blockade with hexamethonium, in one dog MC increased 25%. In three dogs, U46619 ($\mu\text{g/kg}$) caused a mean MC increase of 16.7% (SE ± 7.2). MC increased 16.7% in one dog given U46619 ($1.25 \mu\text{g/kg}$) after ganglionic blockade. In three dogs, U44069 ($\mu\text{g/kg}$) caused a mean MC increase of 16.7% (SE ± 4.2). Two of these dogs were given hexamethonium, following which U44069 in the same dose caused MC increases of 35 and 13%.

Heart rate. The three endoperoxide analogs caused moderate reduction in heart rate during the pressor responses. These changes are as follows: *azo* ($2.5 \mu\text{g/kg}$), 15% in three dogs (SE ± 4.8); U46619 ($1.25 \mu\text{g/kg}$), in six dogs (SE ± 2.4); U44069 (1.25

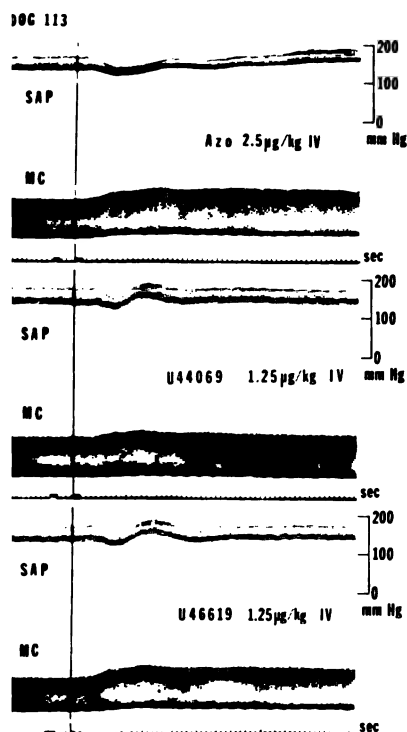
$\mu\text{g/kg}$), 8.9% in seven dogs (SE ± 2.3). Heart rate alterations were reduced or abolished following ganglionic blockade with hexamethonium to 4% for *azo* (one dog); 3% for U46619 (one dog); and no change in heart rate for U44069 (two dogs).

Pretreatment with indomethacin. In two dogs, U46619 and U44069 were given before and after indomethacin (2 mg/kg) which was previously shown in this laboratory to block completely the vascular responses to arachidonic acid. The systemic arterial pressor responses to each of the cyclic ether analogues following indomethacin were as follows: U46619 at $2.5 \mu\text{g/kg}$, 170/135 to 235/195 (38% systolic, 44% diastolic), at $1.25 \mu\text{g/kg}$, 165/130 to 190/155 (15% systolic, 19% diastolic); U44069, $2.5 \mu\text{g/kg}$, 165/135 to 220/185 (33% systolic, 37% diastolic), at $1.25 \mu\text{g/kg}$, 170/135 to 210/170 (23.5% systolic, 26% diastolic).

Comparative studies with $\text{PGF}_{2\alpha}$. Since the endoperoxide analogs were predominantly pressor, $\text{PGF}_{2\alpha}$ ($5 \mu\text{g/kg}$) was given to seven dogs that had received endoperoxide analogs to compare effects (Table I). The mean systolic and diastolic systemic pressure elevations were 15.7 and 17.6%, respectively, (SE ± 4.7 and ± 5.2). Heart rate was reduced during the pressor response, mean 5.2% (SE ± 2.2). PA pressures in three were raised 45, 26, and 62% systolic, and 26, 20, and 242% diastolic (Fig. 1).

Discussion. These first syntheses of stable analogs of PG endoperoxides by Corey *et al.* (5) and by Bundy (6) are of importance to investigators of the PG system, and particularly those who have studied the physiologic and pharmacologic effects of the PG and endoperoxide precursors, since the naturally occurring endoperoxides PGG_2 and PGH_2 are unstable and not generally available. The only information published on the blood pressure effects of PGG_2 and PGH_2 is that these endoperoxides produce a "triphasic response" in the anesthetized guinea pig (7).

The three analogs which were available in limited amounts for this study are extremely active biologically, in some ways contrary to expectations. For example, each is predomi-



2. Simultaneous recordings of SAP and myocontractile force (MC) in a dog receiving the endoperoxide analogs shown. An increase in diastolic contractility occurs, which persists after ganglionic blockade produced by hexamethonium (see

nantly pressor, although the precursor of the bisenoic PGs, AA, is depressor. The slight and transient drop in systemic arterial pressure following intravenous injection of the three analogues, as shown in Figs. 1 and 2, we have interpreted as due to the marked pulmonary vasoconstriction which transiently reduces left ventricular output.

Unlike AA, but similar to the primary PGs, PGE₂ and PGF_{2α}, the three endoperoxide analogs have directly positive inotropic effects on the dog heart (Fig. 2). This response is not blocked by interruption of the baroreceptor reflexes as is the variable myocardial contractile force response to AA.

The molecular manipulations required to make these compounds stable enough for use in studies such as this make it inappropriate to say that the observed responses represent those elicited by the naturally occurring endoperoxides that are formed in the biosynthetic pathway from AA to PGE₂ and PGF_{2α}. In fact, Corey and his colleagues use the expression "highly active biochemical mimic of prostaglandin endoperoxides" in reference to the *azo* compound (5). However, these analogs are potent vasoactive agents and more so than PGF_{2α} which exerts comparable effects on the canine circulation only in larger doses (1, 4). Moreover, these preliminary investigations indicate that the cyclic ether endoperoxide analogs of Bundy are approximately twice as potent in their vasoconstrictor effects, both systemic and pulmonary, as the *azo* analog of Corey and his colleagues.

Summary. Three stable analogs of prosta-

glandin endoperoxides have been studied in limited quantities for their effects on the canine cardiovascular system. They are potent systemic pressor agents and powerful pulmonary vasoconstrictors. They directly increase myocardial contractile force. These responses are not altered by indomethacin. In their blood pressure and myocardial effects they are considerably more potent than arachidonic acid and may be estimated to be more potent than the primary bisenoic PGs, PGE₂ and PGF_{2α}. The cyclic ether endoperoxide analogs of Bundy are approximately twice as potent in their effects on these parameters as the *azo* analog of Corey *et al.*

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Concanavalin A Toxicity: Histological Studies¹ (39513)

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Concanavalin A (Con A), a carbohydrate-metalloprotein that specifically D-glucosyl and sterically related produces vascular endothelial hepatic necrosis, lymphoid atrophy, and 100% mortality within 48 hr after injected intravenously into mice in 0-800 μ g (1). Preliminary ultrastructural studies of livers from mice given 4 hr previously revealed an accumulation of lipid droplets in hepatocytes, degeneration of cell organelle degeneration, most striking, giant mitochondria seemed to be formed as the result of fusion of two or more adjacent swollen mitochondria (2). In this report we will describe a description of the gross, ultrastructural, and histochemical changes found after the injection of 600 μ g of Con A. The histopathological changes noted during a period of 1 year following the iv injection of 200-600 μ g.

Materials and methods. The experimental groups were 12-week-old male B6D2F₁ mice of mice were given 200, 400, or 600 μ g of Con A (Miles Laboratories, Ill.) or saline iv; 6 hr and 1, 2, 7, 14, and 362 days later four experiments from each group and four saline-injected controls were killed and autopsied. The tissues were fixed in 10% neutral formalin and H & E sections were prepared

For electron microscopy, the livers of mice killed 6 hr after injection of 600 μ g of Con A were perfused *in situ* with chilled (4°C) glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.3; the livers

were then removed, cut into thin pieces, and placed in 1.25% glutaraldehyde at 4°C for 30 min.

The method of Bernhard and Avrameas (3) was used for the ultrastructural visualization of Con A. Briefly, the tissues were frozen and sectioned at 1 μ m. They were washed three times in PBS at 4°C and incubated for 30 min at room temperature with type IV horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.) prepared in isotonic saline at a concentration of 50 μ g/ml (horseradish peroxidase is 18% carbohydrate by weight and is bound by Con A already specifically attached to tissue sugar). After three washes in cold PBS, the tissues were fixed in 3% glutaraldehyde for 30 min, and the binding of peroxidase was evaluated by the diaminobenzidine (DAB) method (4). The tissues were postfixed with 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon. Ultrathin sections were examined unstained, or lightly poststained with lead citrate, using a transmission electron microscope, JEOLCO JEM-T7, at 60 kV. Tissues from mice injected with saline were processed in the same manner, and they served as controls.

Results. No deaths occurred among the mice given up to 600 μ g of Con A iv, and at no time did they appear ill. At autopsy, no gross abnormalities were noted among the mice given 200 μ g of Con A. However, histological studies of tissues taken during the 2 days following injection of Con A revealed occasional small areas of periportal necrosis in the livers and mild atrophy of splenic white and red pulp associated with dilated sinusoids and small subcapsular hemorrhages. In addition, the subpleural alveolar capillaries were mildly congested; the renal glomeruli were moderately congested, and the proximal tubules were slightly vacuolated. No significant microscopic abnormalities were found 7, 14, or 28 days after

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the injection of Con A. The remaining animals in this group survived 1 year at which time gross and microscopic findings were within normal limits and indistinguishable from those of the saline-injected controls.

The gross and histological changes produced by 400 and 600 μg of Con A, while somewhat more severe at the higher dose, were similar and, therefore, they will be described together. All experimental mice appeared well 6 hr after the injection of 400–600 μg of Con A. At autopsy, however, the visceral organs showed marked congestion, and there were subcapsular hemorrhages ranging in size from petechial to 5-mm diameter in the spleen, lungs, liver, and kidneys. There was no evidence on gross inspection of hepatic necrosis at this time.

Ultrastructural examination of livers from

mice given saline revealed no abnormalities. In sections treated with peroxidase and DAB, electron-dense material was found in the granules of macrophages and polymorphonuclear cells; in hepatocytes, precipitated material was scattered lightly through the endoplasmic reticulum, and moderately heavy deposits were found along the inner membranes and cristae of mitochondria (Fig. 1). These findings are consistent with the distribution of tissue peroxidases (5).

Examination of hepatocytes from mice given 600 μg of Con A revealed an increase in intracellular lipid droplets, dilation of the endoplasmic reticulum, and mild to marked swelling of mitochondria. The cristae of the enlarged mitochondria were greatly dilated and many were poorly defined. Histochemical studies showed electron-dense material in the granules of macrophages and poly-

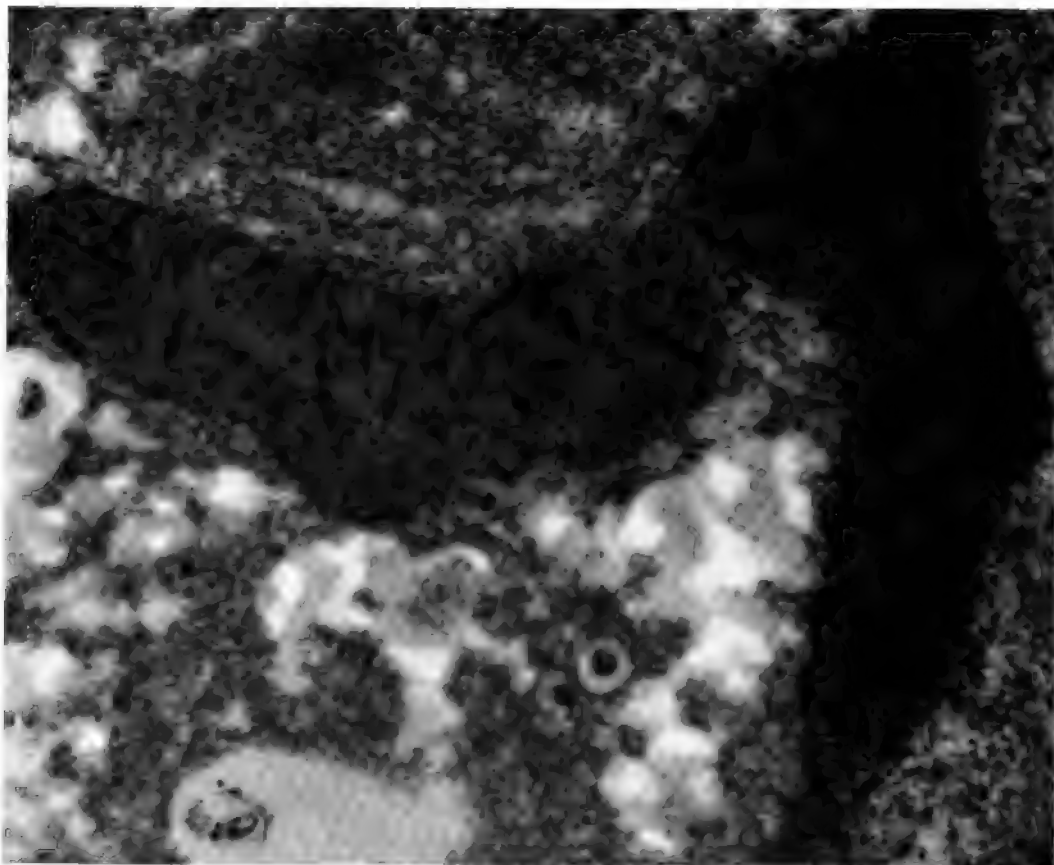


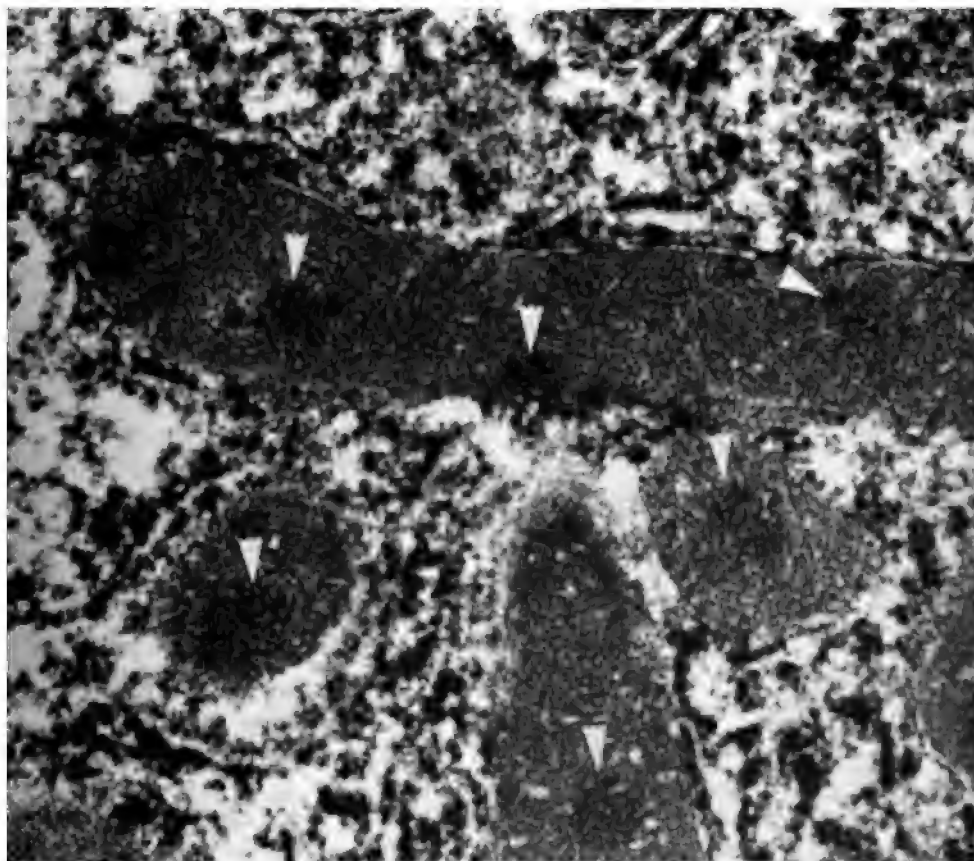
FIG. 1. Liver from a mouse injected with saline. In sections treated with peroxidase and DAB, electron-dense material can be seen in the hepatocytes scattered lightly through the endoplasmic reticulum and in moderately heavy deposits along the inner membranes and cristae of mitochondria. $\times 49,000$.

nuclear cells. Precipitated material and in the dilated endoplasmic reticulum of hepatocytes; there were focal accumulations of electron-dense material within the matrix of swollen mitochondria, but there was an absence of precipitate on the outer and inner mitochondrial membranes (Fig. 1). Heavy deposits of stain were found on the intraluminal surfaces of capillary and dilated endothelial cells, and degenerated endothelial cells were frequently noted (Fig. 2).

Two days after the injection of 400 or 600 μ g of Con A all mice had gross evidence of hepatic damage, and the congestion of subcapsular hemorrhages previously noted in the lungs, spleen, and kidneys were severe. In addition there appeared to be moderate atrophy of the thymus. Micro-

scopically, extensive areas of central and periportal necrosis were observed in the livers; in addition, there was bile stasis and marked congestion of capillaries and sinusoids. The splenic white and red pulp was mildly to markedly atrophic, and there were extensive subcapsular hemorrhages from dilated sinusoids. The thymic cortex was moderately atrophic. The pulmonary alveolar capillaries were congested, and there were large areas of subpleural and alveolar hemorrhage. The renal glomeruli were congested and the proximal convoluted tubules were moderately vacuolated. The following day the gross and microscopic findings were similar although somewhat more advanced (Figs. 4-6).

Seven days after the injection of Con A the livers were finely nodular but much less



2. Liver from a mouse injected 6 hr before with 600 μ g of Con A. The sections were treated with uranyl acetate and DAB. Heavy deposits of electron-dense material are found in the dilated endoplasmic reticulum. Mitochondria are greatly enlarged and the cristae are dilated and poorly defined. Note the absence of stain on mitochondrial membranes and the focal accumulations of dense precipitate within the matrix. $\times 45,000$.

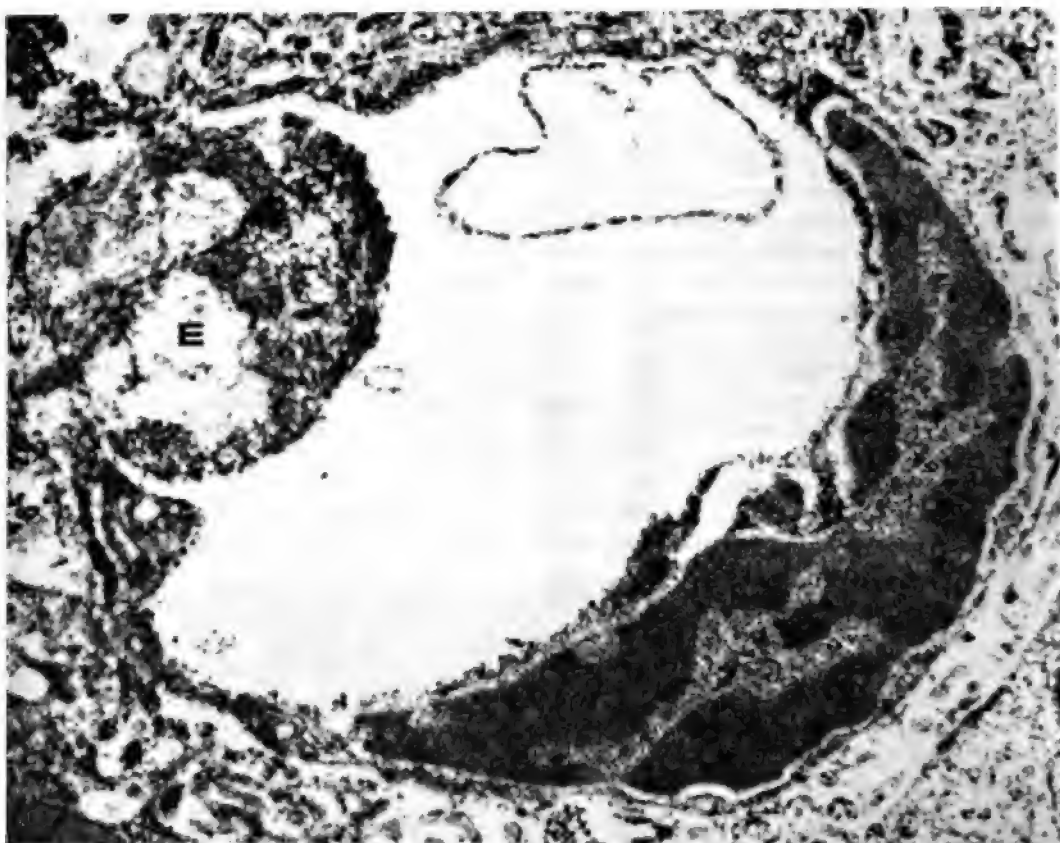
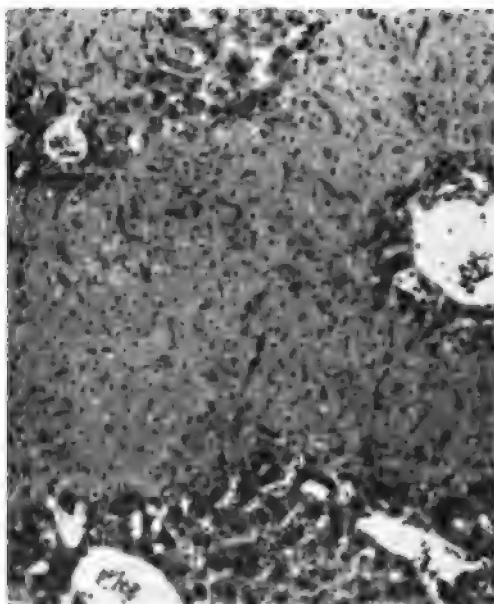
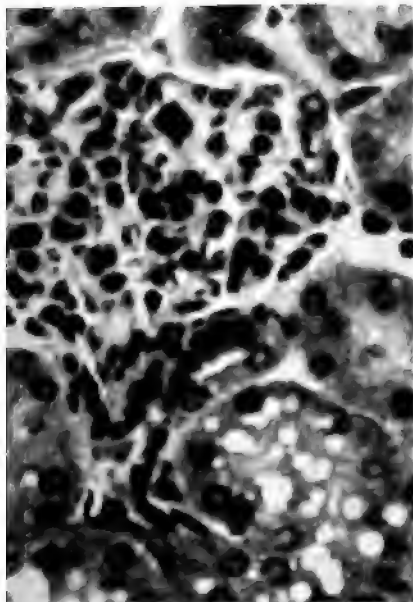


FIG. 3. Hepatic sinusoid of a mouse given 600 μ g of Con A iv 6 hr before. The section was stained with peroxidase and DAB. Electron-dense material lines the entire luminal surface of the endothelial cells, and a degenerating endothelial cell (E) is seen on the left. $\times 23,000$.

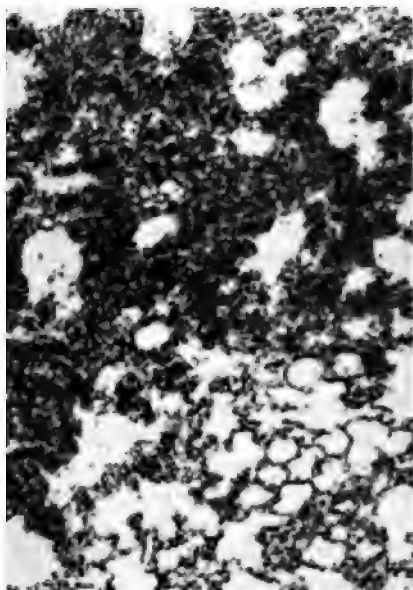


congested. The lungs, spleen, and kidneys remained congested but the majority of the hemorrhages had been resorbed. Microscopically, few necrotic areas were seen in the liver and there was evidence of early regeneration of the hepatocytes. The capillaries and sinusoids were much less congested and focal accumulations of lymphocytes were seen among the hepatocytes. The lungs and spleen remained moderately congested, and there were areas of hemorrhage noted in both organs. In addition, the pulmonary septa were edematous, and there were many small areas of atelectasis. The kidneys were essentially unchanged; the cortical areas of the thymus were hypertrophic.

FIG. 4. H & E section of liver from a mouse given 600 μ g of Con A iv 2 days before. A typical area of massive hepatocellular necrosis is shown. $\times 170$.



H & E section of kidney from a mouse given $600 \mu\text{g}$ Con A iv 2 days before. The glomerulus is in the center and the proximal convoluted tubules are in the periphery. $\times 850$.



H & E section of lung from a mouse given $600 \mu\text{g}$ Con A iv 2 days before. There is marked congestion and many areas of hemorrhage are present. $\times 170$.

Seven days after the injection of Con A, findings on gross examination were similar to those noted 7 days earlier. Microscopically, there was evidence of contin-

uing hepatic regeneration. The vasculature of the lungs, spleen, and kidneys remained congested, and many fibrinous thrombi were noted in pulmonary vessels. The proximal convoluted tubules were vacuolated in some areas; the thymus was normal.

Twenty-eight days after injection the liver appeared normal on gross examination but the lungs and spleen were still somewhat hyperemic. Microscopically, the liver was minimally congested, there were no areas of necrosis, and regeneration was almost complete (Fig. 7). On the other hand, the lungs and kidneys revealed the same vascular changes noted 2 weeks earlier (Figs. 8 and 9). One year after the injection of Con A no significant gross or microscopic changes were noted.

Discussion. These and previous studies suggest that the mechanisms of Con A toxicity in the intact organism and in the isolated cell are complex. It has been shown *in vitro* that within minutes concentrations of Con A as low as $20 \mu\text{g}/\text{ml}$ produce marked changes in the net fluxes of sodium and potassium in mouse tumor cells (6) and that within 2 hours DNA and protein synthesis by mouse thymocytes are decreased and significant

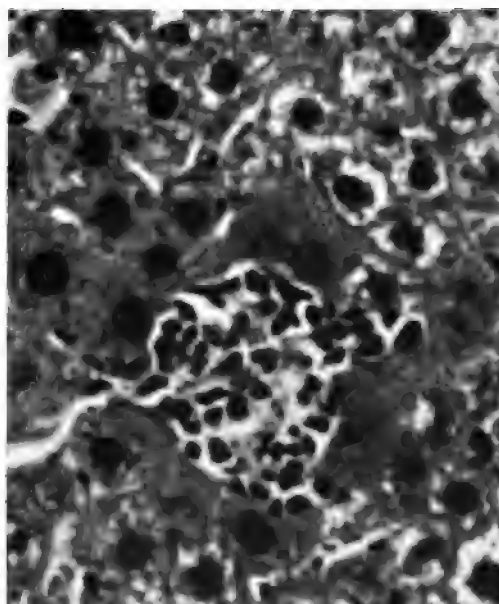


FIG. 7. H & E section of liver from a mouse given $600 \mu\text{g}$ of Con A iv 28 days before. A focal accumulation of lymphocytes is present among the regenerating hepatocytes. $\times 850$.

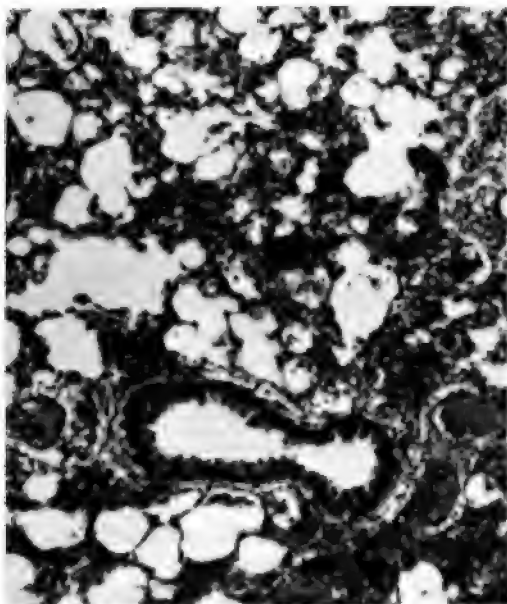


FIG. 8. H & E section of lung from a mouse given 600 μ g of Con A iv 28 days before. There is marked capillary congestion and several fibrinous microthrombi can be seen in vessels adjacent to the bronchus. $\times 170$.

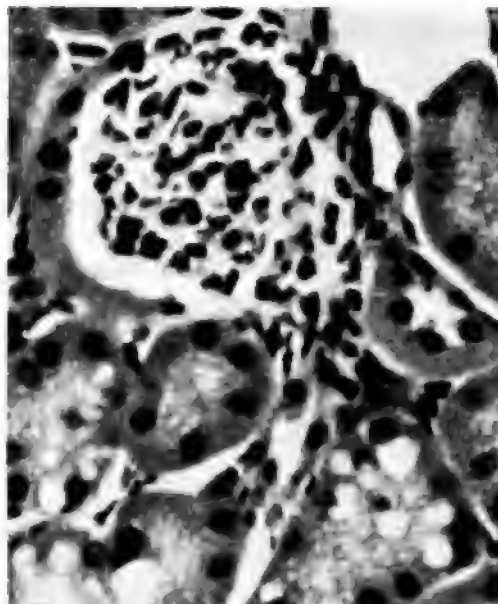


FIG. 9. H & E section of kidney from a mouse given 600 μ g of Con A iv 28 days before. The glomerulus is congested and the proximal tubules are vacuolated. $\times 850$.

cell lysis has occurred (7-9). Taken together these results suggest that Con A impairs cell function by changes produced in the plasma membrane and in various intracellular organelles. The present studies indicate that Con A can interact directly with the endoplasmic reticulum and mitochondria, and that at least a portion of the toxic properties of Con A may result from damage to these organelles.

When Con A was injected intravenously in these experiments, damage to vascular endothelial cells and to hepatic parenchymal cells was found to be well advanced 6 h later. The changes found in the lungs, spleen, kidneys, and liver suggest that damage to the vascular endothelial cells which resulted in thrombosis and hemorrhage may have contributed as much to total tissue damage as did the direct effects of Con A on parenchymal cells. Further, repair of the vascular damage, although apparently complete by 1 year, proceeded at a much slower pace than did hepatic or lymphoid regeneration.

Summary. Con A injected iv in doses of

200-600 μ g produced vascular endothelial damage, hepatic necrosis, and lymphoid atrophy. Six hours after mice were given 600 μ g of Con A iv, subcapsular hemorrhages and other evidence of vascular damage were found in the visceral organs. Ultrastructural studies of livers from these mice revealed damage to the vascular endothelium and to hepatocytes as manifested by the accumulation of lipid droplets, dilation of the endoplasmic reticulum, and the formation of giant degenerating mitochondria. Histochemical studies demonstrated Con A on the cell walls of vascular endothelial cells and in the endoplasmic reticulum and mitochondria of hepatocytes. Con A appeared to destroy the peroxidase activity normally found on the inner membranes and cristae of mitochondria.

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Inhibition of Vitamin D-Stimulated Active Transport of Calcium of Rat Intestine by Diphenylhydantoin-Phenobarbital Treatment (39514)

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The significantly increased incidence of biochemical and radiologic evidence of rickets and osteomalacia in patients receiving anticonvulsants for prolonged periods has been adequately documented (1-3) since the first reports of Schmid (4) and Kruse (5). Lifshitz and MacLaren (6) in an epidemiological study in an institution for severely retarded patients have shown that the patients on anticonvulsant drugs had lower serum calcium and phosphate concentrations and higher alkaline phosphatase activities than those with comparable motor disabilities who were not receiving anticonvulsants. This effect was seen primarily in patients receiving both phenobarbital and diphenylhydantoin. Inasmuch as phenobarbital is known to induce the liver microsomal enzymes of the cytochrome *P*-450 system which hydroxylates steroids which are then glucuronidated (7), the possibility that anticonvulsant medication has an anti-vitamin D effect through increasing the rate of inactivation of 25-OH-vitamin D has been proposed (8). MacLaren and Lifshitz (9) have treated institutionalized patients with anticonvulsant-associated rickets using physiological amounts of 25-OH-cholecalciferol and have demonstrated healing in patients who were unresponsive to much larger amounts of ergocalciferol. They suggested that the anticonvulsants interfered with 25-hydroxylation by liver.

Studies in rats have suggested an additional effect of diphenylhydantoin which might contribute to the increased vitamin D requirement of patients on anticonvulsant therapy. No evidence of an anti-vitamin D action of diphenylhydantoin in rats has been demonstrated using skeletal changes of rickets (10) or hypocalcemia as an indicator (unpublished data). However, Caspary (11) and Koch *et al.* (12) have reported impairment of intestinal calcium transport in rats

given diphenylhydantoin or diphenylhydantoin plus phenobarbital.

The present studies were designed to examine this effect and to determine whether it could be separated from a deficiency of the active vitamin D metabolite, 1,25-dihydroxyvitamin D, resulting from the action of the anticonvulsants. The design of the experiments was based on the measurement of response of hypocalcemic, vitamin D-depleted rats treated with diphenylhydantoin and phenobarbital to physiological amounts of vitamin D or 25-hydroxy-vitamin D using three criteria of response: serum calcium concentration, active transport of calcium *in vitro* by everted intestinal loops, and diffusibility of calcium across the intestinal wall *in vitro*. The active transport of calcium in the *in vitro* system is indicated by the development of a concentration of calcium in serosal fluid much higher than that in mucosal fluid and is an energy-requiring process. The diffusion of calcium from a higher concentration in the mucosal phase to a lower concentration in the serosal phase occurs at low temperature and in the presence of metabolic inhibitors which block active transport of calcium. Vitamin D increases both diffusibility and active transport of calcium across the intestinal mucosa. If a separation of the effect of vitamin D on these two processes could be demonstrated in the diphenylhydantoin- and phenobarbital-treated rats, the locus of action of the anticonvulsant drugs on calcium transport might be further delineated. The combination of diphenylhydantoin and phenobarbital was used to duplicate more closely the clinical state in man since the increased susceptibility to rickets and osteomalacia is most striking in patients receiving combinations of anticonvulsant drugs usually including diphenylhydantoin (6, 13).

Methods. Weanling male rats of the

zman strain were fed a vitamin D-deficient diet containing 0.6% calcium and 0.6% phosphorus. On this diet, vitamin D deficiency is not associated with hypophosphemic rickets but with hypocalcemia.

After 3 weeks on the diet the rats were bled from the tail and serum calcium concentrations determined to verify the state of vitamin D depletion. The animals were then divided into groups. Treated rats were given 100 mg diphenylhydantoin, 8 mg/100 g, and 100 mg phenobarbital, 5 mg/100 g, daily by peritoneal injection divided in two doses, and treatment was continued until the animals were killed. Control rats were given equivalent volumes of 0.85% saline solution intraperitoneally. The food intake and weight gain of treated and control rats were measured and the anticonvulsant drug treatment did not reduce food intake or weight gain. On the fifth day of such injections the rats were given by stomach tube 100 µg (625 pmoles) of ergocalciferol, cholecalciferol, or 25-OH-cholecalciferol dissolved in propylene glycol. Groups of treated and control rats given only solvent were also studied. One series of treated and control rats was given a larger dose of cholecalciferol, 2.5 µg (6.25 nmoles). Seventy-two hours after the steroid administration the rats were anesthetized with sodium pentobarbital and bled from the abdominal aorta, and segments of duodenum and ileum were taken for the measurement of active calcium transport *in vitro* by an everted intestinal loop system (15). The buffer solution in the calcium transport system contained 50 mequiv/liter of sodium since at

this concentration of sodium both ileal and duodenal transport of calcium are maximal (16).

In another series of experiments the diffusibility of calcium across the intestinal wall was measured, also by use of everted intestinal loops of duodenum or ileum. These determinations were done at room temperature (23°). In mucosal fluid the initial concentration of calcium labeled with calcium-45 was 2.5 mM, with no calcium in serosal fluid. The accumulation of calcium-45 in serosal fluid during a 30-min incubation was determined. The effect of vitamin D in increasing net mucosal to serosal movement of calcium under these conditions is not blocked by inhibition of metabolic energy production (15, 17), and this system may measure facilitated diffusion of calcium across the brush border of the mucosal cell. The treatment of the animal was the same as in the first series of experiments. Serum calcium concentrations were measured by atomic absorption spectrophotometry (18) and phosphate concentrations by the Fiske and Subbarow method (19).

Results. Table I summarizes the response of serum calcium and phosphate concentrations of diphenylhydantoin- and phenobarbital-injected and control vitamin D-depleted rats to treatment with ergocalciferol, cholecalciferol, and 25-OH-cholecalciferol. Dilantin/phenobarbital administration did not alter the concentrations of these ions in the serum of vitamin D-deficient rats nor did it modify the increase of serum calcium concentration following administration of the various forms of vitamin D. Table II

TABLE I. SERUM CALCIUM AND PHOSPHATE CONCENTRATIONS IN CONTROL AND ANTICONVULSANT-TREATED RATS.

Concentration in serum (mg/100 ml)	No vitamin D		EC* (0.25 µg)		CC* (0.25 µg)		CC (2.5 µg)		25-OHCC* (0.25 µg)	
	C*	D-P*	C	D-P	C	D-P	C	D-P	C	D-P
Calcium	5.3 ^a ± 0.20 (6)*	5.6 ± 0.19 (5)	7.3 ± 0.56 (6)	7.7 ± 0.40 (7)	7.6 ± 0.30 (5)	7.9 ± 0.13 (9)	9.6 ± 0.12 (6)	9.8 ± 0.19 (5)	7.8 ± 0.27 (5)	8.4 ± 0.23 (9)
Phosphorus	8.7 ± 0.27 (6)*	8.4 ± 0.35 (5)	8.1 ± 0.30 (6)	8.2 ± 0.30 (7)	8.2 ± 0.17 (5)	7.8 ± 0.19 (9)	10.8 ± 0.25 (6)	10.4 ± 0.34 (6)	9.0 ± 0.20 (5)	8.4 ± 0.25 (9)

*Ergocalciferol; CC, cholecalciferol; 25-OHCC, 25-hydroxycholecalciferol.

control, saline injected.

D-P, diphenylhydantoin and phenobarbital injected.

mean ± SEM.

*, Number of rats in group.

TABLE II. EFFECT OF DIPHENYLHYDANTOIN/PHENOBARBITAL ADMINISTRATION ON *in Vitro* TRANSPORT OF CALCIUM BY EVERTED SEGMENTS OF RAT SMALL INTESTINE.^a

Calcium	No vitamin D		EC (0.25 μ g)		CC (0.25 μ g)		CC (2.5 μ g)		25-OHCC(0.25 μ g)	
	C	D-P	C	D-P	C	D-P	C	D-P	C	D-P
Duodenum	1.58 \pm 0.18 (6)	1.57 \pm 0.20 (4)	4.16 * \pm 0.75 (6)	2.20 \pm 0.37 (5)	5.61 * \pm 1.52 (5)	2.58 \pm 0.20 (8)	6.94 * \pm 1.64 (5)	1.76 \pm 0.18 (6)	5.08 * \pm 0.74 (9)	2.29 \pm 0.31 (9)
Ileum	1.00 \pm 0.03 (6)	1.00 \pm 0.09 (4)	4.16 \pm 1.27 (6)	2.51 \pm 0.36 (7)	3.75 \pm 0.61 (5)	2.66 \pm 0.45 (9)	10.50 * \pm 1.45 (6)	5.73 \pm 0.63 (6)	4.77 * \pm 0.78 (9)	2.51 \pm 0.34 (9)

^a All of the data represent C_s/C_m values, i.e., the ratio at the end of incubation of the concentration of ion in the serosal fluid to that in mucosal fluid. The degree to which this ratio is increased above 1, the initial state, represents the net mucosal to serosal transport of the ion. The incubation period for the calcium transport studies was 90 min. The values are means \pm SEM. An asterisk (*) between a pair of values indicates a significant difference ($P < 0.05$). (n), Number of everted loops on which mean is based.

presents the results of the active calcium transport measurements expressed as C_s/C_m values. The diphenylhydantoin plus phenobarbital injection did not further reduce the low calcium transport rates in duodenum or ileum of vitamin D-depleted rats. This treatment did, however, significantly reduce the effect of 0.25 μ g of ergocalciferol, cholecalciferol, and 25-OH-cholecalciferol on calcium transport by duodenum as well as the effect of the larger dose of cholecalciferol, 2.5 μ g. Although ileal calcium transport was somewhat less in the treated rats receiving 0.25 μ g of ergocalciferol in comparison with controls, the differences were not statistically significant. The decreases were significant in the groups receiving either the larger dose of cholecalciferol, 2.5 μ g, or 25-OH-cholecalciferol.

The results of the second type of experiment in which the non-energy-requiring diffusibility of calcium across the intestinal wall was measured are shown in table III. The diffusion of calcium into the serosal compartment was increased by treatment of the vitamin D-depleted rats with 0.25 μ g of cholecalciferol, and this effect was not significantly altered by injection of the rats with diphenylhydantoin and phenobarbital. In this group of rats the increase of serum calcium concentration following vitamin D was actually greater in the rats given anticonvulsants than in the saline-injected controls, but this was not found in the other series of experiments.

Discussion. The significant inhibition by

TABLE III. EFFECT OF DIPHENYLHYDANTOIN/PHENOBARBITAL TREATMENT ON DIFFUSIBILITY OF CALCIUM ACROSS INTESTINAL WALL *in Vitro*.

Calcium diffusion ^a (μ g Ca/30 min/loop)	No vitamin D C	CC (0.25 μ g)	
		C	D-P
Duodenum	3.08 \pm 0.17 (11)	8.02 \pm 1.00 (14)	6.28 \pm 0.54 (14)
Ileum	1.33 \pm 0.17 (11)	2.22 \pm 0.20 (14)	2.52 \pm 0.18 (14)
Serum Ca (mg/100 ml)	5.4 \pm 0.16 (11)	7.7 * \pm 0.24 (14)	8.6 \pm 0.14 (14)

^a The everted loops were incubated at 23° for 30 min.

diphenylhydantoin/phenobarbital treatment of the vitamin D-induced increase of metabolically dependent calcium transport by rat intestine in these *in vitro* experiments cannot be explained in terms of reduced concentrations of the active vitamin D metabolite unless it is postulated that the effect of vitamin D on active transport of calcium requires higher concentrations of vitamin D than its other effects. There is no inhibition of the serum calcium raising effect of the steroid which also presumably involves the formation of the active metabolite, 1,25-diOH-cholecalciferol. The interaction of the active vitamin D metabolite with the intestinal epithelial cell is apparently not blocked by the anticonvulsant drugs since the vitamin D effect on calcium diffusibility across

cosal barrier is not diminished. This is the observation of Koch *et al.* (12) and that treatment with diphenylhydantoin reduced rat duodenal calcium transport *in situ* but did not inhibit the vitamin D-induced increase of calcium-binding protein in the duodenal mucosa. It is possible that the effect of calcium diffusibility across the intestinal wall is related to the increase of calcium-binding protein following vitamin D treatment. Since these two effects of vitamin D on the intestinal epithelium are not blocked by diphenylhydantoin or the diphenylhydantoin/phenobarbital combination it would seem that the reduction of calcium transport by anticonvulsants is at a more distal site than the calcium transport system. If it is realized that one function of vitamin D-induced calcium-binding protein is to accelerate the flow of calcium from mucosal across the brush border surface of the intestinal cell, the inhibitory action of diphenylhydantoin and phenobarbital could be at some phase of the linkage of metabolism with the energy-dependent ejection of calcium at the basal surfaces of the cell. It is this latter action in which increases the concentration of calcium in the fluid in contact with the apical pole of the cell. How this action of diphenylhydantoin on intestinal calcium transport is related to its pharmacologic action on the central nervous system or conduction system is only conjectural. Inhibition of diphenylhydantoin *in vitro* to offer a solution in which the intestinal mucosa were incubated was made to determine whether a direct inhibitory effect on active transport would occur. In these experiments active calcium transport was measured in everted duodenal loops from rats pretreated with 0.5 μ g of cholecalciferol 72 hr earlier. In everted loops, diphenylhydantoin was added to the buffer in a final concentration of 1 mM. The average C_s/C_m ratios following 90 min of incubation were 6.78 and 1.0 for control and treated duodenal loops. It is possible that the diphenylhydantoin did not enter the intestinal mucosal cells during the period of incubation or that the inhibitory effect is not due to this compound but to a metabolic derivative or to alteration of another component following prolonged exposure to the drug.

In man, anticonvulsant combinations such as diphenylhydantoin and phenobarbital do have actions on vitamin D metabolism since there is evidence of reduced concentrations of 25-OH-cholecalciferol in patients treated with these drugs (20). However, Jubiz *et al.* (21) have recently reported that 1,25-diOH-cholecalciferol concentrations in the serum of anticonvulsant-treated patients were not reduced despite lower concentrations of 25-OH-cholecalciferol. They concluded that the disturbances of calcium metabolism of patients treated with anticonvulsants cannot be explained by a deficiency of the active vitamin D metabolite. The possibility that diphenylhydantoin may reduce intestinal calcium absorption in man also by a mechanism analogous to the *in vitro* or *in situ* inhibition found in the rat remains to be determined. If such calcium malabsorption due to diphenylhydantoin does occur, this could explain hypocalcemia and secondary hyperparathyroidism in the presence of normal concentrations of 1,25-diOH-vitamin D. Such a diphenylhydantoin action might also be more significant in the immobile, bedridden patient who seems to be at greater risk for bone disease associated with anticonvulsant medication.

Summary. The effect of combined diphenylhydantoin and phenobarbital administration on the response of vitamin D-depleted rats to ergocalciferol, cholecalciferol, and 25-hydroxycholecalciferol (25-OHCC) was measured by determinations of serum calcium and phosphate concentrations and of intestinal transport of calcium *in vitro* by everted loops of small intestine. The major action of this anticonvulsant drug treatment in the rat was to inhibit the action of the various forms of vitamin D including 25-OHCC in increasing active transport of calcium by the everted intestine. The increase of calcium diffusibility across the intestine by vitamin D was not blocked nor was the action of vitamin D in increasing serum calcium concentrations. A specific inhibitory effect of the anticonvulsant drugs on an energy-dependent calcium transport system in the intestinal mucosa is suggested.

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Effects of Neurotransmitter Receptor Antagonists on Ether-Induced Prolactin Release in Ovariectomized, Estrogen-Treated Rats¹ (39515)

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Termination of the neurotransmitter regulate ether-induced prolactin release under current investigation in our series. Numerous reports have indicated ether increases plasma prolactin (10), but little is known about the neurotransmitter systems in the central nervous system that are involved in this release. The administration of nicotine has been shown not to affect the ether-induced release of prolactin (9), whereas blockade of adrenergic receptors with methysergide or stimulation of dopaminergic receptors with apomorphine (3) block this release. Evidence exists that ether influences adrenergic systems in the rat brain; however, reserpine treatment failed to alter ether-induced prolactin release in male rats (13). The purpose of this study was to determine if the antagonism of several neurotransmitter systems plays any role in the secretion of prolactin in ether-anesthetized, ovariectomized, estrogen-treated

Materials and methods. Sixty mature, female Sprague-Dawley rats (Spartan Research Animals, Inc., Haslett, Mich.) weighing 180 to 260 g were randomly divided into 10 groups. After 6 to 7 days of adaptation to lighting conditions (lights on 0600 to 2000 hr) the rats were bilaterally ovariectomized. Seven to 14 days after ovariectomy 0.5 mg of polyestradiol acetate (PEP; 1 mg of Estradurin, Ayerst Laboratories, Inc.) was administered to each rat subcutaneously and 7 days later a second injection at 0900 the following experimental day was initiated. All animals except one group (uninjected controls) were ether-anesthetized and injected intraperitoneally with

vehicle (saline or 95% EtOH) or drug. The dosage of each drug except the histaminergic antagonists was based on previous studies (18, 19). The dosage of each of the histaminergic antagonists was based on information supplied by the manufacturers. Twenty-five minutes after injection all animals were anesthetized by an initial exposure to ether vapor in a large container followed by maintenance with a nose cone. Ether anesthesia once initiated was maintained throughout the duration of the experiment. Blood samples (0.5 ml) were taken 5, 15, 25, and 35 min after ether exposure (30, 40, 50, and 60 min after injection) via orbital sinus puncture and transferred to tubes containing 0.5 ml of heparinized phosphate-buffered saline (50 units of heparin/ml). The diluted plasma was collected and stored at -20°C until assayed.

The drugs employed in this study were: phenoxybenzamine-HCl (a gift from Smith, Kline and French Laboratories, Philadelphia, Pa.), phentolamine-HCl (Regitine, a gift from Ciba Pharmaceutical Co., Summit, N.J.), propranolol-HCl (Inderal, a gift from Ayerst Laboratories, Inc., New York, N.Y.), 2-chloro-2'-[3-dimethylamino]propyl]thio-cinnaminilide-HCl (SQ 10,631, a gift from E. R. Squibb and Sons, Inc., Princeton, N.J.), metiamide-HCl (a gift from Smith, Kline and French Laboratories, Philadelphia, Pa.), pyrilamine maleate (generously provided by Merck, Sharp and Dohme, Rahway, N.J.), and methysergide maleate (a gift from Sandoz Pharmaceutical Division, Hanover, N.J.). All drugs were given intraperitoneally at a dose of 10 mg/kg, except SQ 10,631 and methysergide, which were given at 2.5 mg/kg.

Plasma samples were assayed by double antibody radioimmunoassay (14) in duplicate, each at two dilutions. Rat prolactin NIAMDD-RP-1 (11 IU/mg), supplied through the Rat Pituitary Hormone Distri-

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bution Program of the National Institute of Arthritis, Metabolism and Digestive Diseases, was used as the standard.

One-way analysis of variance (15) was used to assess statistical significance between the vehicle-injected control and experimental groups at each time period.

Results. The response of prolactin to ether anesthesia was significantly ($P < 0.025$) elevated by handling and/or injection of vehicles compared to non-handled uninjected controls. Plasma prolactin levels in saline- and ethanol-injected animals,

however, were not different from each other at any of the time periods examined (Fig. 1A). The administration of α -adrenergic blocking drugs, phenoxybenzamine and phentolamine, significantly enhanced the ether-induced elevation in prolactin at 5 and/or 15 min but not at 25 or 35 min of ether anesthesia (Fig. 1B). In contrast, β -adrenergic blockade with propranolol had little to no effect on ether-induced prolactin release (Fig. 1C).

The administration of H_1 and H_2 histaminergic blockers, pyrilamine and metiamide,

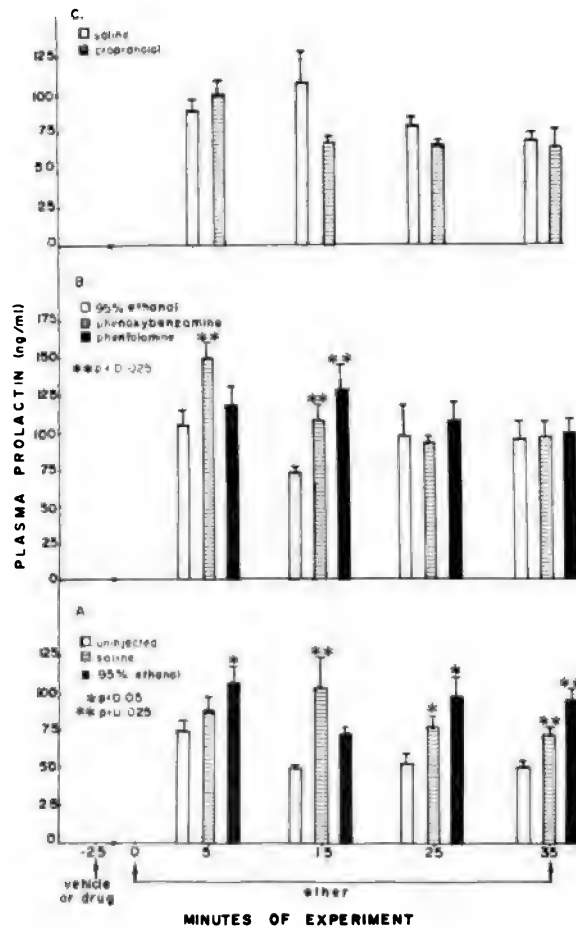


FIG. 1. (A) Comparison of plasma prolactin levels of uninjected and vehicle-injected ovariectomized, estrogen-treated rats during ether anesthesia. Values represent the mean \pm SEM of six animals in each group. (B) Effect of α -adrenergic antagonists, phenoxybenzamine and phentolamine, at 10 mg/kg ip on plasma prolactin levels in ovariectomized, estrogen-treated

rats during ether anesthesia. Values represent the mean \pm SEM for six animals in each group. (C) Effect of a β -adrenergic antagonist, propranolol, at 10 mg/kg ip on plasma prolactin levels in ovariectomized, estrogen-treated rats during ether anesthesia. Values represented the mean \pm SEM for six animals in each group.

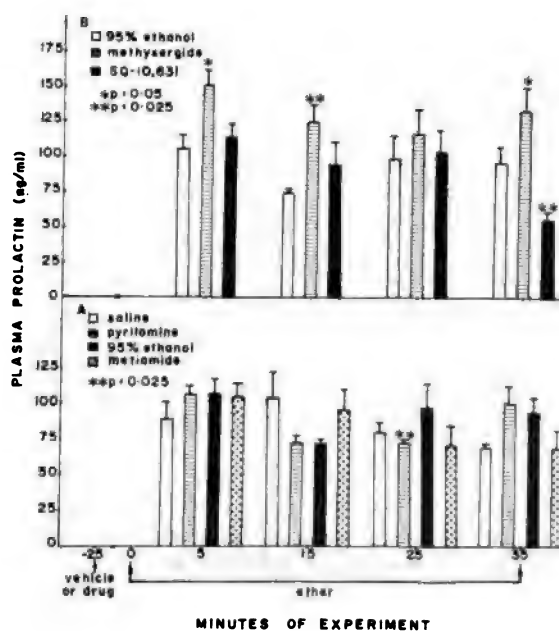
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to blunt the ether-induced release of prolactin but only pyrilamine showed a significant ($P < 0.025$) suppression at 25 min after anesthesia when compared to saline-anesthetized controls (Fig. 2A).

Methysergide and SQ 10,631, two serotonin antagonists, had different effects on ether-induced prolactin response. Methysergide significantly ($P < 0.025$) increased prolactin secretion initially (5 and 15 min) as SQ 10,631 significantly ($P < 0.05$) reduced the prolactin response at 35 min after anesthesia (Fig. 2B).

Discussion. The data presented in this review indicate that the ether-induced increase in plasma prolactin in ovariectomized, estradiol-treated rats can be altered by non-hormonal factors such as handling and intraperitoneal injection of vehicles and by the peritoneal administration of α -adrenergic, dopaminergic, and serotonergic antago-

nists. The inhibitory dopaminergic influence on prolactin release (3). It is possible that ether may directly inhibit dopamine transmission in the hypothalamo-hypophyseal axis or indirectly affect dopamine transmission by stimulating other pathways that have inhibitory effects. The ether enhances [3 H]norepinephrine release from the brain (12) may act by an indirect mode of action. Whether ether has such an action in selectively affecting the hypothalamus which controls prolactin secretion is unknown. The present results show that the ether response is significantly enhanced by α -adrenergic blockade with phenoxybenzamine and phentolamine (1B), indicating that noradrenergic influences, like dopaminergic systems, are antagonistic to ether-induced prolactin release. However, these agents may have some overlapping blockade of dopamine receptors. Dopamine antagonism has been demonstrated for phentolamine (13) and Leod and Lehmeyer (16) at the level of the pituitary.



2. (A) Effect of H₁- and H₂-histaminergic antagonists, pyrilamine and metiamide, respectively, at 1 mg/kg ip on plasma prolactin levels in ovariectomized, estrogen-treated rats. Comparisons were made between pyrilamine and saline and between metiamide and saline. (B) Effect of serotonin antagonists, methysergide and SQ 10,631 at 1 mg/kg ip on plasma prolactin levels in ovariectomized, estrogen-treated rats. The values represent the mean \pm SEM for six animals in each group.

6. (A) Effect of H₁- and H₂-histaminergic antagonists, pyrilamine and metiamide, respectively, at 1 mg/kg ip on plasma prolactin levels in ovariectomized, estrogen-treated rats. Comparisons were made between pyrilamine and saline and between metiamide and saline. (B) Effect of serotonin antagonists, methysergide and SQ 10,631 at 1 mg/kg ip on plasma prolactin levels in ovariectomized, estrogen-treated rats. The values represent the mean \pm SEM for six animals in each group.

To the authors' knowledge this is the first report to show a role of histaminergic neurotransmission in ether-induced prolactin release. Libertun and McCann (17) have shown that intraventricular histamine elevates basal levels of prolactin and that diphenylhydramine, an H_1 -receptor antagonist, blocks this rise and also the rise induced by restraint. In the present investigation pyrilamine maleate, a fairly selective H_1 -histaminergic receptor blocker, significantly blunted the ether-induced rise in prolactin whereas metiamide, a selective H_2 -histaminergic blocker, had little to no effect on the ether-induced response.

The decrease in ether-induced prolactin release produced by serotonergic receptor blockade in females supports in general the observations of Marchlewska-Koj and Kruulich (11) in males. The delayed inhibitory effect of SQ 10,631 on the ether-induced response was similar to that recently reported by us for pimozide-induced prolactin release (18). The stimulatory effect of methysergide observed here is in contradiction to a recent report that methysergide blocked the ether-induced increase in prolactin (11). This apparent conflict may be accounted for by a sex difference or by a difference in drug dosage (2.5 vs 50 mg/kg). The importance of a sexual difference in the prolactin response to methysergide is supported by the observations that methysergide induced no change in baseline values in males (11) but caused increased baseline values in females (19, 20). Indeed, this prolactin-releasing effect of methysergide in the female may be responsible for the elevated ether-induced response noted in the early time periods in the present study. These comments on sex and dose differences notwithstanding, SQ 10,631 and methysergide may produce different effects on prolactin secretion because of different mechanisms of blockade at the serotonin receptor. It is also possible that multiple types of serotonin receptors may exist in the CNS.

These pharmacologic studies by no means demonstrate unequivocally that the ether-induced prolactin response involves inhibition of hypothalamic α -adrenergic neurons or stimulation of H_1 -histaminergic and serotonergic neurons, but do warrant more ex-

tensive studies along these lines. The additional possibility that other nonspecific stresses such as handling and/or intraperitoneal injections can enhance the ether-induced response indicates the need for future work on the nature of the pathways over which different types of stresses increase prolactin release. The nonspecific effects of the drugs over and above the effects of handling and injection also cannot be completely discounted. In other studies, however, changes in blood pressure as a result of the injection of several drugs were not correlated with changes in prolactin release (unpublished observations). This suggests that the alterations in prolactin release observed in this study were probably not due to nonspecific stresses induced by the drugs.

Summary. The role of various neurotransmitter receptor antagonists in the ether-induced release of prolactin in estrogen-treated ovariectomized rats was investigated. Serial blood samples were obtained by orbital sinus puncture at 5, 15, 25, and 35 min of continuous ether anesthesia. Animals were pretreated 25 min before the onset of anesthesia with either α - or β -adrenergic, H_1 - or H_2 -histaminergic, or serotonergic antagonists. α -Adrenergic blockade increased the ether-induced release of prolactin during the early phase of ether anesthesia as did treatment with methysergide, a 5-HT antagonist. Blockade of H_1 -histaminergic receptors and treatment with another 5-HT antagonist, SQ 10,631, decreased the ether-induced response in the later phase of anesthesia. β -Adrenergic and H_2 -histaminergic blockade were without effect on the ether-induced release of prolactin. These studies indicate the possible involvement of noradrenergic, histaminergic, and serotonergic systems in the release of prolactin following ether anesthesia in the female rat.

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Activity of Selected 2,4-Diaminoquinazolines against *Candida albicans* in Vitro¹ (39516)

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Several years ago a broad spectrum *in vitro* antimicrobial screening program was conducted in these laboratories employing a wide variety of quinazoline derivatives (1). One compound, 6-(3,4-dichlorophenylacetamido)-2,4-diaminoquinazoline (structure A, Table I) was found to display selective inhibitory effects against *Candida albicans* in this qualitative test system. Interestingly, this compound was originally synthesized as a potential antimalarial agent by Hynes and Ashton (2). It was found to be inactive against *Plasmodium berghei* in mice as well as *Plasmodium gallinaceum* in chicks and also to be nontoxic to both host species. Based upon this information, it was suggested that A may be effective against other fungi such as *Cryptococcus neoformans*. It is well known that the chemotherapy of systemic infections due to this organism is far from satisfactory since only amphotericin B and 5-fluorocytosine are of value, and each of these drugs has major limitations (3). A recent communication by Hariri and Larsh confirmed this hypothesis since A was highly effective against *C. neoformans* in mice (3). Unfortunately, the source of the earlier results from our laboratories was inadvertently omitted from their paper. The current study was conducted to ascertain the structure-activity patterns for compounds related to compound A against *C. albicans*.

Materials and methods. The 2,4-diaminoquinazolines (compounds A-I) were available by virtue of a recent synthetic study (2).

5-Fluorocytosine was obtained from Roche Laboratories. Incubation flasks for *C. albicans* were standard 50-ml Erlenmeyer flasks with uniform-bore 19-mm-o.d. tubes fused near the tops. Tilting of the contents of each flask permitted measurement of the optical density (OD) of the contents. The strict linearity of the OD as a function of cell population was confirmed by appropriate dilutions of a late log phase culture followed by OD measurements; no attempt was made in this preliminary study to differentiate a fungistatic effect from a fungicidal effect. Each flask contained 19.8 ml of sterile Sabouraud dextrose broth (Difco) and 0.2 ml of dimethylsulfoxide (DMSO) containing varying concentrations of each quinazoline compound to yield the desired final concentrations. Control flasks contained DMSO at the same final concentration. 5-Fluorocytosine was dissolved in sterile water. Inoculum was 0.2 ml of an overnight culture of *C. albicans* in Sabouraud broth. After 8 and 24 hr of incubation at 37° on a Dubnoff reciprocating shaker, the OD of each culture was measured at 640 nm with a Coleman spectrophotometer, and the concentration of each compound which conferred 50% inhibition of growth (IC₅₀) was determined graphically. Acute toxicity tests of compounds A and D were done in Sch:ARS HA(ICR)₁ mice (Sprague Dawley). A saline suspension of each compound was treated for 15 sec with a Branson Sonifier to yield a fine suspension. Groups of six mice received 25, 100, or 250 mg/kg of each compound ip; the concentration was adjusted so that each mouse received 1.0 ml of suspension per 30 g of body weight. Mean weights of all groups were recorded daily.

Results and discussion. As shown in Table 1, several different structural modifications

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re evaluated for activity against *C. albicans*. The results obtained with 5-fluorocytosine are included for comparison. Modifications included alteration of the aryl group (B, C, and D), modification of the bridge between the aryl moiety to the 6-position of the quinazoline nucleus (B, C, and D), insertion of a methyl or chloro group at position 5 (H and I). For the compounds studied, it is apparent that the 3,4-diphenyl group affords optimal activity. Finally, the elongation of the aryl group by a methylene group (C) or insertion of a small hydrophobic group at the 5-position is deleterious to activity. However, the

excision of one methylene unit (D) results in a compound significantly more active than A and which rivals 5-fluorocytosine in effectiveness.

Based upon these results, compounds A and D were selected for acute toxicity studies and the results are summarized in Table II. It will be seen that A caused no fatalities even at 250 mg/kg. On the other hand, D produced significant weight losses at 100 mg/kg and at the highest dose tested one (of six) animals failed to survive. In spite of its close structural similarity to known inhibitors of dihydrofolate reductase, compound D was found to be an exceptionally poor inhibitor of the rat liver enzyme, while A was reasonably effective (2). Therefore, it

TABLE I. *In Vitro* ACTIVITY OF 6-SUBSTITUTED-2,4-DIAMINOQUINAZOLINES AGAINST *Candida albicans*.

Id No.	R ₅	Y	Ar	IC ₅₀ (μg/ml)					
				8-Hr growth Isolate No.			24-Hr growth Isolate No.		
				I	II	III	I	II	III
	H	$\begin{array}{c} \text{O} \\ \parallel \\ \text{NHCCH}_2 \end{array}$	3,4-Cl ₂ C ₆ H ₃	0.7	3.8	2.3	2.5	5.3	3.3
	H	$\begin{array}{c} \text{O} \\ \parallel \\ \text{CH}_2\text{NHC} \end{array}$	3,4-Cl ₂ C ₆ H ₃	5.8	8.0	>15.0	12.8	14.5	9.5
	H	$\begin{array}{c} \text{O} \\ \parallel \\ \text{CH}_2\text{NHCCH}_2 \end{array}$	3,4-Cl ₂ C ₆ H ₃	6.5	13.5	>15.0	>15.0	>15.0	>15.0
	H	$\begin{array}{c} \text{O} \\ \parallel \\ \text{NHC} \end{array}$	3,4-Cl ₂ C ₆ H ₃	0.8	0.6	0.6	2.0	3.6	1.0
	H	$\begin{array}{c} \text{O} \\ \parallel \\ \text{NHCCH}_2 \end{array}$	3-(CF ₃)C ₆ H ₄	6.3	15.0	8.4	11.8	>15.0	9.8
	H	$\begin{array}{c} \text{O} \\ \parallel \\ \text{NHCCH}_2 \end{array}$	4-ClC ₆ H ₄	3.5	4.5	>15.0	10.5	10.8	6.8
	H	$\begin{array}{c} \text{O} \\ \parallel \\ \text{NHCCH}_2 \end{array}$	2-C ₁₀ H ₇	3.0	2.8	13.8	6.8	10.0	4.6
	Cl	$\begin{array}{c} \text{O} \\ \parallel \\ \text{NHCCH}_2 \end{array}$	3,4-Cl ₂ C ₆ H ₃	7.0	7.3	5.4	14.3	>15.0	13.5
	CH ₃	$\begin{array}{c} \text{O} \\ \parallel \\ \text{NHCCH}_2 \end{array}$	3,4-Cl ₂ C ₆ H ₃	5.6 0.5	7.4 0.4	>15.0 1.2	12.5 2.7	13.8 0.7	12.5 3.0

TABLE II. ACUTE TOXICITY OF SELECTED QUINAZOLINE DERIVATIVES IN MICE.

Compound	Dose (mg/mg)	Average weight (g) ^a Days postinjection								
		0	1	2	3	4	5	6	7	8
A	25	26.9	27.3	27.4	27.7	27.8	—	28.5	28.5	28.3
A	100	27.1	25.8	26.7	27.3	27.8	—	28.6	29.0	28.9
A	250	26.0	23.8	25.3	25.8	26.0	—	27.6	27.5	27.2
D	25	27.8	25.3	24.7	25.3	26.3	—	27.5	27.7	27.2
D	100	29.9	27.8	24.8	25.8	26.7	—	26.8	26.5	26.8
D	250	27.6	25.8	23.3	21.7	21.5	—	21.6 ^b	21.8 ^b	22.0 ^b

^a Six mice per group.^b One mouse died; average weight of five remaining mice.

appears that the toxicity of D is not related to the inhibition of this metabolic transformation. In any event, neither A nor D displayed toxicity at potentially useful therapeutic levels.

In view of these results, *in vivo* studies have been initiated with these two compounds against a variety of pathogenic fungi. In addition, new structures will be synthesized in an effort to achieve higher levels of antifungal activity.

Summary. A series of nine 2,4-diaminoquinazolines bearing a variety of substituents at positions 5 and 6 was evaluated against three human isolates of *Candida albicans in vitro*. An established drug, 5-fluorocytosine, was chosen as a standard of comparison and each isolate was found to be

sensitive to this agent. The two most active compounds, 6-(3,4-dichlorophenylacetamido)-2,4-diaminoquinazoline and 6-(3,4-dichlorobenzamido)-2,4-diaminoquinazoline, were selected for acute toxicity studies in mice. At the highest dose tested (250 mg/kg) the latter compound showed evidence of toxicity while both compounds were substantially free of untoward effects at lower doses.

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Serum Potassium and Sodium Concentrations of Rat Blood Obtained by Different Methods¹ (39517)

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Serum K^+ values are frequently used in biologic studies. Hypokalemia resulting, respectively, from a deficiency of mineralocorticoids, is a highly useful index of adrenal function. It is also sometimes useful to measure plasma renin activity by assaying mineralocorticoid hormones. This is best done by rapid decapitation to avoid stress-induced hypokalemia. In our hands the K^+ values of serum are inordinately elevated, doubtless due to the contribution of intracellular potassium from ruptured somatic cells.

It has been reported that blood from the severed tail tip of the rat by the method of Enta (2) yields plasma potassium values within the normal range, but at this might prove to be a cumbersome procedure.

A method which should yield reliable values is that of the negligible degree of contamination therewith is that of cardiac puncture (3, 4). This has, however, several disadvantages. Anesthesia is required, the procedure is time consuming, and until skillful operators are frequently killed. The study was designed to compare serum K^+ obtained by these various

methods. These studies were conducted on Sprague-Dawley SPD, Wistar WKY and COBS rats. Blood was collected either by rapid guillotine, by clipping a small piece of the rat tail (under ether anesthesia although this is not an essential), or by cardiac puncture of anesthetized rats. Blood was allowed to clot and was then centrifuged to serum. Potassium values were determined by means of a flame photometer

by Grant HL 09911 from the U. S. Service, National Heart and Lung Institute.

(Beckman Kline Flame). Statistical comparisons were made using Student's two-tailed t test.

Results. Experiment 1. This experiment employed groups of young female SPD and Fischer 344 rats that had received one 40-mg pellet of deoxycorticosterone acetate (DOCA) by subcutaneous implantation 73 days prior to sacrifice or had served as unimplanted controls. No other treatment had been given, and the animals had received tap water and Purina laboratory chow *ad lib*. At sacrifice the rats were decapitated within 10 sec of removal from their cages. Trunk blood was collected into plastic dishes and allowed to coagulate. Serum Na^+ and K^+ values are given in Table I.

Experiment 2. In this experiment eight Wistar WKY rats which had not been subjected to experimentation, but had been maintained on tap water and Purina laboratory chow were used. Under ether anesthesia, blood was collected first by cardiac puncture and immediately thereafter from the clipped tail. Serum Na^+ and K^+ values are shown in Table II.

Experiment 3. Since the previous experiment had indicated that, while tail blood K^+ values were within the range obtained by others (1), the values were nevertheless higher than simultaneously drawn heart blood, an experiment was undertaken to determine whether a second sample drawn after an interval had elapsed from the first collection would yield truer values.

Eight Wistar COBS rats, which had not been under treatment but had been maintained on normal food and water in the colony, were used. Under ether anesthesia, freely flowing blood was collected from a severed tail tip. Two additional drops were allowed to fall and were discarded. A second collection was then made. The serum K^+ values are shown in Table III.

Discussion. Serum from blood collected

TABLE I. SERUM Na⁺ AND K⁺ CONCENTRATIONS IN DOCA-TREATED AND CONTROL SPD AND FISCHER 344 RATS.

Data	SPD		Fischer 344	
	DOCA	Controls	DOCA	Controls
N	8	8	9	8
Serum K ⁺	6.55 ± 0.20 ^{a, b}	7.64 ± 0.21	6.12 ± 0.13 ^b	7.13 ± 0.10
Serum Na ⁺	124.12 ± 0.81	122.62 ± 0.65	125.33 ± 1.40	122.50 ± 0.78

^a Mean ± SEM. Values are given as milliequivalents per liter.^b Significantly different from control values (*P* < 0.002).TABLE II. SERUM Na⁺ AND K⁺ CONCENTRATIONS OF HEART AND TAIL BLOOD.

Data	Heart blood	<i>P</i>	Tail blood
N	8		8
Serum Na ⁺	143.25 ± 0.65 ^a	N.S.	143.50 ± 1.11
Serum K ⁺	4.91 ± 0.07	<0.0001	6.32 ± 0.16

^a Mean ± SEM. Values are given as milliequivalents per liter.TABLE III. SERUM Na⁺ AND K⁺ CONCENTRATIONS IN SEQUENTIALLY DRAWN SAMPLES OF TAIL BLOOD.

Data	First sample	Second sample
N	8	8
Serum Na ⁺ (mEq/liter)	142.35 ± 0.54 ^a	142.97 ± 0.62
Serum K ⁺ (mEq/liter)	5.76 ± 0.28	5.50 ± 0.10

^a Mean ± SEM. Values are given as milliequivalents per liter.

by cardiac puncture had the lowest and least variable K⁺ concentration and a normal Na⁺ concentration. Simultaneously collected tail blood yielded serum with a comparable Na⁺ concentration, but a significantly higher K⁺ concentration. Without exception serum K⁺ values were 1.1 to 2.1 mequiv/liter lower from heart than from tail blood of the same animal. In the second experiment, where extreme care was taken to clip as little of the tail tip as possible in order to prevent undue contamination of blood with extravascular fluid, the serum Na⁺ values were quite similar to those obtained by that method previously. Serum K⁺ values were somewhat lower and at the upper limit of such values reported by other investigators (1). However, they were somewhat higher than the earlier values obtained by cardiac puncture, and it was evident that no improvement was attained by collecting a second sample after allowing an interval to elapse after collect-

ing the first. In one instance the K⁺ values were identical in both samples, in three the first sample was slightly lower than the second, and in four the reverse obtained.

Trunk blood from decapitate animals gave serum that had diminished Na⁺ and elevated K⁺ concentrations. It was evident that the samples had been significantly contaminated with intracellular fluid high in K⁺ and low in Na⁺.

"Serum" K⁺ concentrations from decapitate rats were, in fact, high enough to have been toxic had they actually occurred. Despite these distortions, the mineralocorticoid effect of DOCA was clearly discernible. Hormone-treated groups had serum Na⁺ concentrations that were about 2 mequiv/liter lower and K⁺ concentrations about 2 mequiv/liter higher than those present in untreated groups, although in both cases the Na⁺ concentration was about 20 mequiv/liter lower than in blood obtained from the heart or tail. This change was sufficient to produce a highly statistically significant depression of serum K⁺ in DOCA-treated rats, but the hypernatremia was not great enough to do so because of the much higher normal values for the concentration of that ion.

In general, it appears that elevation of serum K⁺ is a reliable index of the degree of cellular trauma incident to blood collection. Although only blood obtained by cardiac puncture appears to yield entirely reliable K⁺ values, those obtained from the severed tail tip often fall within an acceptably "normal" range, and even the most unreliable concentrations, those present in trunk blood from decapitate rats, could be used to demonstrate the hypokalemic effects of a potent mineralocorticoid hormone.

Summary. Blood obtained from anesthetized rats by cardiac puncture, severance of

the tail tip, or decapitation, was analyzed for serum Na^+ and K^+ . The lowest, least variable, and hence most reliable serum K^+ values were obtained from heart blood. Tail-blood samples gave serum K^+ values which, while often in a plausible range, were substantially higher. Trunk blood collected from decapitates gave inordinately high serum K^+ and low Na^+ values. These, while obviously inaccurate, could still be used to demonstrate the hypokalemic effects of mineralocorticoid hormone. If accuracy is essential, cardiac puncture is the only reliable

method among those used for obtaining serum K^+ .

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Inhibition of *in Vitro* Neutrophil Chemotaxis and Spontaneous Motility by Anti-Inflammatory Agents¹ (39518)

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Acutely inflamed tissues are rich in neutrophils and current evidence suggests that chemotactic stimuli may be responsible for the entry of this cell into sites of inflammation (1, 2). Many anti-inflammatory agents are available which dampen the inflammatory response. One possible mode of action of these drugs could be to modulate the response of the neutrophil to chemotactic stimuli. In order to test this hypothesis we undertook to study *in vitro* the effect of anti-inflammatory agents on the responsiveness of rabbit and rat peritoneal and human blood neutrophils to chemotactic material obtained from *Escherichia coli* culture filtrates. To study the specificity of these agents on chemotactic stimuli this study also investigated the effect of anti-inflammatory agents on neutrophil spontaneous motility.

Materials and methods. Drugs were obtained from the following sources: triamcinolone acetonide, triamcinolone acetonide dipotassium phosphate, halcinonide, cicloprofen, procainamide, and niflumic acid from Squibb; diazepam from Roche; hydrocortisone succinate, prednisolone, and methyl prednisolone from Upjohn; hydrocortisone acetate and indomethacin from Merck; cytoxan from Mead Johnson; imuran from Burroughs-Wellcome; chloroquine phosphate from Winthrop; ibuprofen from Boots Pure Drug; phenylbutazone from Ciba-Giegy; naproxen from Syntex; aspirin from Monsanto; sudoxicam from

Pfizer; ethynylestradiol and progesterone from Schering.

Peritoneal exudates containing 85-95% neutrophils were induced in female New Zealand white rabbits with 0.02% shellfish glycogen (Sigma) as previously described (3). Rat peritoneal exudates, also 85-95% rich in neutrophils, were induced by injecting 20 ml of 12% sterile sodium caseinate (DIFCO) into the peritoneal cavity and aspirating the exudate 24 hr later through the use of an 18-gauge needle. Human blood neutrophils 94-100% pure were obtained by the Ficoll-Hypaque method (4) from healthy males and females who had not taken any medication for at least 1 month prior to donation of the blood.

The neutrophils obtained from the above three sources were washed and suspended at a concentration of 5×10^6 cells/ml in Hanks' balanced salt solution, pH 7.2, containing 0.02 M Tris buffer and 0.2% bovine serum albumin (BSA, Fraction IV, Sigma). The test drugs were dissolved at twice their final concentration in the above buffer without BSA, the pH readjusted to 7.2 if necessary, and the solution diluted 1:1 with the cell suspension so that the final cell concentration was 2.5×10^6 /ml in 0.1% BSA. This cell suspension was added directly to the chemotaxis chambers without a preincubation step unless otherwise indicated. The control consisted of a cell suspension free of drug but containing the vehicle used to dissolve the test drug.

The chemotactic agent for the rabbit and rat neutrophil experiments was a butanol extract of an *E. coli* culture filtrate, diluted 1/8000 with buffer when used with rabbit neutrophils, and 1/500 when used with rat neutrophils. The crude supernatant of the above *E. coli* culture filtrate diluted 1/25 served as the positive chemotactic factor in the human blood neutrophil experiments.

Chemotaxis and spontaneous motility

¹ This communication was given in preliminary form at the 59th Annual Meeting of the Federation of American Societies for Experimental Biology, April 13-18, 1975.

² Some of the data presented here have been taken from a dissertation submitted by C. H. Rosen in partial fulfillment of the requirements for the degree of Bachelor of Arts, Department of Biology, Princeton University.

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measured by the Boyden chamber as previously described (5). For axis experiments a Millipore filter of $0.8\text{-}\mu\text{m}$ average pore size divided the chemotaxis chamber into the upper and lower compartments. Cell suspension with or without added drug was placed in the upper compartment immediately after injecting bacterial chemotactic factor into the lower compartment. Each experiment was run in triplicate along with duplicate chambers that contained only buffer in the lower compartment for assessment of background activity. The chambers were incubated for 90 min at 37° , and the filters were removed and histologically stained for microscopic scoring at $40\times$ magnification. The number of random fields of each filter were counted consisting of all the cells that had migrated into the filter from the initial top layer. The mean activity in the three replicates from each experimental condition was determined, and the background activity was subtracted. The final results, unless otherwise indicated, are reported as the molar concentration of drug which inhibited chemotaxis by 50% (I_{50}).

Spontaneous motility was measured in triplicate exactly as outlined above except that a $5\text{-}\mu\text{m}$ average pore size Millipore filter was used and only buffer was placed in the bottom compartment.

None of the drugs used in this study affected neutrophil viability as determined by the ability of the cells to exclude eosin-Y.

Results. Table I demonstrates that both steroidal and nonsteroidal anti-inflammatory agents inhibit neutrophil chemotactic responsiveness to bacterial chemotactic factor. Except for indomethacin in the human neutrophil system, the inhibition of chemotaxis observed with a particular drug was consistent. Each drug reported in Table I was assayed at least twice. Phenylbutazone was assayed twice with human blood neutrophils with identical I_{50} values being observed each time. This drug was assayed another four times with rabbit neutrophils and each time there was significant inhibition of chemotaxis at the concentrations tested. Prednisolone at a concentration of $1 \times 10^{-5} M$ was employed in each experiment as a positive drug control. This drug also has consistently inhibited chemotaxis.

TABLE I. EFFECT OF ANTI-INFLAMMATORY AGENTS ON NEUTROPHILS FROM RABBIT AND RAT PERITONEAL EXUDATES AND HUMAN BLOOD.

Drug ^a	Neutrophil source ^b		
	Rabbit	Rat	Human
Phenylbutazone succinate	4×10^{-6}	2×10^{-6}	5×10^{-6}
Phenylbutazone acetate	No effect	NT ^c	NT
Prednisolone	4×10^{-6}	5×10^{-7}	NT
Indomethacin	4×10^{-6}	NT	1×10^{-7}
Prednisolone acetonide	1×10^{-5}	4×10^{-6}	4×10^{-6}
Prednisolone acetonide dipotassium	$>10^{-3}$	1×10^{-4}	6×10^{-3}
Aspirin			
Sodium salicylate	7×10^{-5}	NT	3×10^{-5}
Acetylsalicylic acid	2×10^{-5}	1×10^{-4}	1×10^{-5}
Sodium salicylate	7×10^{-5}	7×10^{-5}	7×10^{-5}
Phenylbutazone	3×10^{-4}	1×10^{-6}	NT
Indomethacin	1×10^{-6}	3×10^{-4}	6×10^{-5}
Phenylbutazone	9×10^{-7}	3×10^{-7}	1×10^{-5}
Indomethacin	1×10^{-5}	NT	5×10^{-6}
Phenylbutazone	5×10^{-6}	NT	2×10^{-5}
Indomethacin	4×10^{-7}	NT	2×10^{-7}
Aspirin	1×10^{-2}	1×10^{-2}	2×10^{-4}
Phenylbutazone PO_4^{d}	No effect	NT	No effect
Indomethacin ^d	No effect	No effect	Variable

^aagents were mixed with the cells and immediately added to the chemotaxis chambers and assayed as described in Materials and Methods.

^bes are reported as I_{50} (M), the molar concentration of drug which inhibited chemotaxis by 50%.

^ctested.

^dest concentration tested = $1 \times 10^{-4} M$.

The data in Table I also demonstrate that the two immunosuppressive agents, imuran and cytoxan, inhibit chemotaxis.

The steroidal anti-inflammatory agent hydrocortisone succinate significantly inhibited neutrophil chemotaxis as shown in Table I, whereas the acetate of hydrocortisone had no effect at concentrations up to $1 \times 10^{-3} M$. Note also that the anti-inflammatory steroid triamcinolone acetonide inhibited chemotaxis whereas its phosphate ester was effective only at very high concentrations. It should be pointed out that diazepam and procainamide, drugs totally unrelated to anti-inflammatory agents, were tested and found to have no effect on neutrophil chemotaxis. The steroid hormones progesterone and ethinylestradiol were tested on human blood neutrophils and found to have no effect.

Indomethacin, at concentrations up to $1 \times 10^{-4} M$, had no effect on rabbit and rat neutrophil chemotaxis even when preincubated with the cells for $\frac{1}{2}$ hr at 37° prior to their addition to the chemotaxis chambers. This is shown in Table II with rabbit peritoneal neutrophils where it can be seen that indomethacin at the concentrations tested did not inhibit chemotaxis even upon preincubation with the cells. Similar negative results were obtained with rat peritoneal neutrophils. With respect to the human neutrophil system, indomethacin at concentrations below $1 \times 10^{-4} M$ had no appreciable effect on chemotaxis; at $1 \times 10^{-4} M$ indomethacin gave variable results.

Aspirin, when added to neutrophil suspensions and placed immediately in the chemotaxis chamber, inhibited chemotaxis of rat and rabbit peritoneal neutrophils only at extremely high concentrations (Table I). However, Fig. 1 illustrates that preincubation of aspirin with rabbit peritoneal neutrophils for $\frac{1}{2}$ hr at 37° prior to their addition to the chemotaxis chamber increases inhibition of chemotaxis 100-fold. The I_{50} for aspirin was $2 \times 10^{-2} M$, however, it increased to $2 \times 10^{-4} M$ when the drug was in contact with the cells prior to their addition to the chemotaxis chambers. Similar results, not shown here, were obtained using rat neutrophils. It should be pointed out that preincubation of neutrophils with triamcinolone ac-

TABLE II. LACK OF EFFECT OF INDOMETHACIN ON RABBIT PERITONEAL NEUTROPHIL CHEMOTAXIS.

Drug	Experimental condition ^a	
	No preincubation	$\frac{1}{2}$ -Hr preincubation
Control		
No drug added	140 ± 2	120 ± 12
Indomethacin		
$1 \times 10^{-6} M$	149 ± 8	107 ± 3
$1 \times 10^{-5} M$	138 ± 10	110 ± 11
$1 \times 10^{-4} M$	126 ± 8	109 ± 10
Prednisolone		
$1 \times 10^{-5} M$	63 ± 3	47 ± 3

^a Test agents were mixed with the cell suspensions and either added immediately to the upper compartment of the chemotaxis chamber or incubated for $\frac{1}{2}$ hr at 37° and then added to the chambers. The chambers were then incubated for 90 min at 37° and the activity determined as indicated in Materials and Methods. The chemotactic activity is reported as cells per three high-powered fields minus background activity \pm SEM.

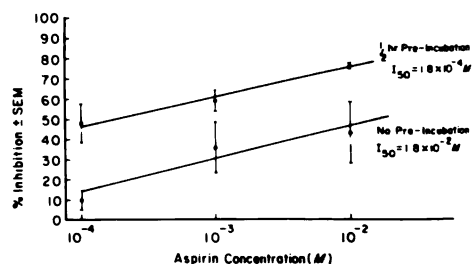


FIG. 1. Inhibition of chemotaxis by aspirin. The drug was mixed with cell suspension (rabbit peritoneal neutrophils) and either added immediately to the upper compartment of the chemotaxis chambers or incubated for $\frac{1}{2}$ hr at 37° and then added to the chambers. The chambers were then incubated for 90 min at 37° and the activity determined as indicated in Materials and methods.

etonide dipotassium phosphate did not improve its ability to inhibit chemotaxis.

Table III presents our findings with respect to the effect of anti-inflammatory agents on neutrophil spontaneous motility. Similar results were obtained with rabbit peritoneal neutrophils; rat neutrophils were not used in the motility studies. The data in Table III demonstrate that the anti-inflammatory agents fall into two categories with respect to their effect on neutrophil movement. All the steroidal anti-inflammatory agents tested affected neutrophil spontaneous motility at concentrations which inhibited chemotaxis (Table I). The immunosuppressive agents cytoxan and imuran also

TABLE III. EFFECT OF ANTI-INFLAMMATORY AGENTS ON HUMAN BLOOD NEUTROPHIL SPONTANEOUS MOTILITY.

Drug ^a	Spontaneous motility I_{50} (M) ^b
Hydrocortisone	4×10^{-5}
Prednisolone acetate	5×10^{-5}
Hydrocortisone succinate	4×10^{-5}
Phenylbutazone	2×10^{-4}
Aspirin	1×10^{-3}
	2×10^{-4}
Indomethacin	3×10^{-4}
Chloroquine	No effect
Chloroquine phosphate	No effect
Triamcinolone	No effect
Triamcinolone acetate	No effect
	No effect

agents were mixed with the cells and immediately added to the chemotaxis chamber for the measurement of spontaneous motility. A 5- μ m Millipore filter was used and only buffer placed in the bottom compartment of the chamber as indicated in Materials and Methods.

^a = the molar concentration of drug which inhibited spontaneous motility by 50%.

of spontaneous motility (Table III). Furthermore, the nonsteroidal anti-inflammatory agents tested, with the exception of phenylbutazone, specifically inhibited the chemotactic responsiveness of the neutrophils at concentrations that had no effect on spontaneous motility.

Discussion. This report is the first demonstration that a wide variety of anti-inflammatory drugs inhibits the *in vitro* chemotactic responsiveness of the neutrophil. This is specific for anti-inflammatory and suppressive agents. Drugs not considered anti-inflammatory or immunosuppressive agents had no effect on chemotaxis. These results are in agreement with those reported by Ward (6, 7) for hydrocortisone, prednisolone, phenylbutazone, and aspirin. He also reported that chloroquine inhibited rabbit peritoneal neutrophil chemotaxis. We tested chloroquine phosphate (the chloride salt was not available) and found it to be inactive.

Chloroquine phosphate ester of triamcinolone acetate was found to be active only at very low concentrations (Table I), whereas the free molecule was considerably more active. This is in keeping with our findings with chloroquine phosphate and suggests that the phosphate group renders the mole-

cule impermeable to the cell membrane. The acetate derivative of hydrocortisone was found to be inactive, whereas hydrocortisone itself effectively inhibited neutrophil chemotaxis. This is in keeping with the report that hydrocortisone acetate is impermeable to leukocyte membranes (8).

Our results (and those of Ward (6, 7)) are in contrast to those reported by Borel (9) who tested seven anti-inflammatory agents for their effect on rabbit peritoneal neutrophil chemotaxis and found no effect with phenylbutazone and naproxen and variable effects with hydrocortisone succinate and aspirin. The reason or reasons for these discordant results are essentially unknown. Conditions under which neutrophil migration were assayed might well explain the divergence in results between Borel and ourselves. We are currently investigating this problem in an attempt to resolve the reasons for our different findings with respect to these agents.

Indomethacin was found to have no significant effect on *in vitro* rabbit and rat neutrophil chemotaxis and had a variable effect on human neutrophil chemotaxis, the reasons for which are currently under investigation. Similar results were reported by Borel (9) using rabbit neutrophils. Indomethacin is a potent inhibitor of carrageenin-induced rat paw edema (10) and adjuvant-induced arthritis in rats (11) and is used clinically as an effective anti-inflammatory agent. Our findings with respect to indomethacin and neutrophil chemotaxis suggest that the anti-inflammatory activity of this agent may possibly not entail a direct effect on neutrophil migration.

On the other hand, many of the anti-inflammatory agents listed in Table I were found to inhibit *in vitro* neutrophil chemotaxis at concentrations that may have *in vivo* clinical significance (12-15). This suggests that one of the possible mechanisms of their anti-inflammatory activity is to inhibit neutrophil migration. We are currently studying an *in vivo* model of chemotaxis to investigate the significance of these *in vitro* findings.

Our data also demonstrate that, for most of the agents we tested, the source of the neutrophil is not critical for the observation

of a drug effect on chemotaxis; an effective agent inhibited neutrophil chemotaxis from rabbit and rat peritoneal exudates and human blood and an ineffective agent had no effect on neutrophils from any of these three sources.

This study has shown also that with respect to neutrophil chemotaxis the anti-inflammatory agents tested, with the exception of phenylbutazone, fell into two categories. The effective steroidal anti-inflammatory agents which were tested inhibited both chemotaxis and spontaneous motility, whereas the effective nonsteroidal agents tested inhibited only chemotaxis. It is interesting to note that aspirin which inhibited neutrophil chemotaxis, albeit at very high concentrations, had no effect at all on neutrophil motility, even at concentrations as high as $1 \times 10^{-2} M$. The two immunosuppressive agents tested, cytoxan and imuran, inhibited both chemotaxis and motility. These results suggest that the effect of steroidal anti-inflammatory and immunosuppressive agents on neutrophil chemotaxis is probably due to the effect of these agents on the cell's spontaneous motility. On the other hand, the nonsteroidal anti-inflammatory agents may act on parameters concerned with the ability of the neutrophil to respond to chemotactic stimuli.

Summary. A number of anti-inflammatory agents were tested for their effect on the chemotaxis responsiveness and on the spontaneous motility of neutrophils obtained from rabbit and rat peritoneal exudates and human blood. The majority of these agents, both steroidal and nonsteroidal, inhibited the chemotactic responsiveness of neutrophils obtained from all three sources to a bacterial chemotactic factor. The effective steroidal anti-inflammatory agents tested inhibited both chemotaxis and

spontaneous motility, whereas the nonsteroidal agents, with the exception of phenylbutazone, inhibited only chemotaxis and were without effect on motility.

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Parotid Salivary Protein Present during Late Pregnancy and Postpartum¹ (39519)

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... provides a potential means for de-
and investigation of physiologic
s which occur during and after preg-
Differences from nonpregnant indi-
in the concentration of salivary cal-
sodium, and phosphorus were ob-
in normal pregnancy (1, 2). A de-
in flow rate and an increase in protein
tration of parotid saliva have also
ected in pregnant subjects (2). Un-
apparently there are no published
of changes in specific salivary pro-
luring pregnancy and postpartum.
per describes a change in a human
salivary protein or proteins ob-
during late pregnancy, immediately
tum, and 4-6 weeks postpartum de-
by polyacrylamide-gel electrophore-

ods. Approximately 10 ml of stimu-
arotid saliva (sour lemon drops) were
ed using a Teflon suction cup device
m eight pregnant subjects at each
visit (Obstetrics and Gynecology
University of Minnesota), when per-
by the clinic schedule, from about 2-
ths gestation to 2 weeks prepartum
4-6 weeks postpartum. Samples were
ed from three of the above eight do-
months postpartum. Parotid saliva
llected from an additional individual
1 of the 9 days prior to delivery and 1,
3 days postpartum. Single samples
otained 1-4 days postpartum from 55
subjects. In addition, parotid saliva
llected by the same technique as used
e above subjects from 12 male do-
id 12 female subjects not pregnant
previous 12 months. Flow rates and
concentration determined by the
ure of Lowry *et al.* (4) were recorded
otid saliva samples collected from
nt subjects at various times during
ncy and postpartum. The salivary

specimens were dialyzed immediately after
collection against several changes of dis-
tilled water at 4° for 48 hr and lyophilized.
Multiple samples from the same subject
were stored after lyophilization until all ex-
cept the 4 months postpartum samples had
been collected.

Each lyophilized residue was reconsti-
tuted to 10 mg/ml in 0.9% NaCl. The pro-
teins in duplicate 30- μ l aliquots of these
solutions were separated by electrophoresis
at pH 9.0 on 15-cm-wide, 2-mm-thick poly-
acrylamide gel slabs, 3.0 cm of 6% gel over
7.5 cm of 10% gel according to a previously
described technique (5). Electrophoresis
was conducted at 4° and at 40 mA constant
current (150-200 V) until bromphenol blue
marker dye reached the bottom of the gel
(approximately 3.5 hr). All samples from a
donor, except the 4 month postpartum spec-
imens, were placed on the same gel. Pro-
teins in the gels were fixed by immersion of
the gels in 50% trichloroacetic acid for 30
min and were stained for 30 min with 0.1%
Coomassie brilliant blue in 50% trichloroa-
cetic acid. Excess stain was removed with
several changes of 10% glacial acetic acid.
The preparations were subjected to contin-
ual mechanical shaking during fixing, stain-
ing, and destaining. The stained salivary
protein patterns were recorded by photo-
graphing the gels under standardized con-
ditions. In addition, a portion of the samples
was scanned with an Ortec Model 4310 den-
sitometer. Reproducibility of the above
technique has been verified and discussed in
previous publications (6, 7).

Results. The flow rate (single gland) and
protein concentration of parotid saliva from
pregnant subjects were (mean \pm SD) 1.02 ± 0.42 ml/min and 154 ± 69 mg/100 ml,
respectively. No consistent and significant
change in flow rate and protein concentra-
tion was noted for individual subjects during
pregnancy and postpartum.

Figure 1 gives the parotid salivary protein
patterns obtained from all samples collected

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15322.

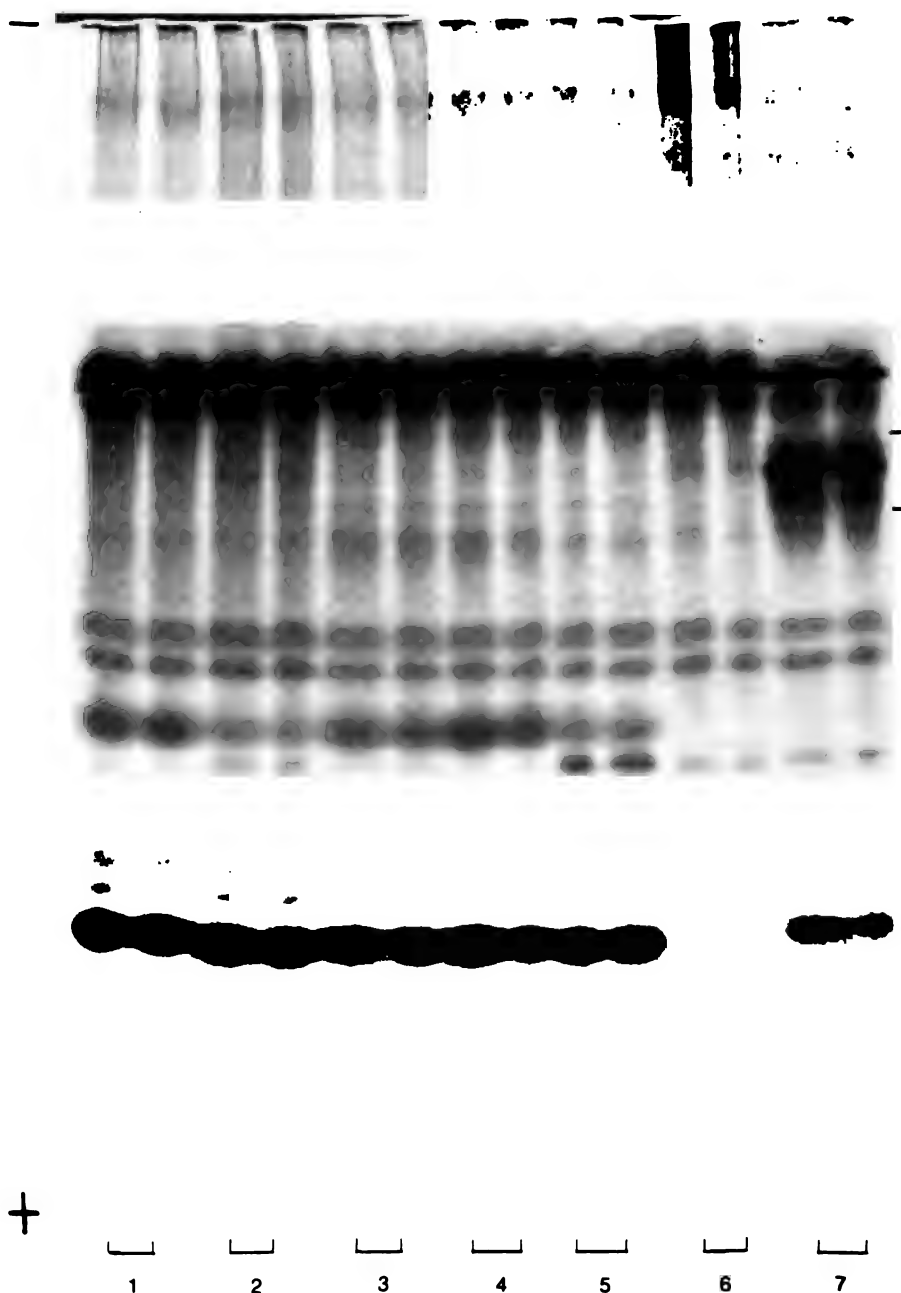


FIG. 1. Polyacrylamide gel slab patterns of parotid salivary proteins collected from one subject during pregnancy and postpartum. Each sample was run in duplicate on adjacent channels. Sample 7, located at the right margin of the gel, was collected 4 weeks postpartum. Samples obtained from 2 months gestation (sample 1) to 6 weeks prepartum (sample 6) are placed in sequence of collection from the left margin of the gel to the sample adjacent to the postpartum specimen. The electrophoretic zone containing pregnancy-associated protein is indicated by the bracket on the right margin of the figure. Anode (+) and cathode (-) are indicated on the left margin of the gel.

e subject. A large increase in Coomassie-staining material is evident in the gel about one-third the distance from the top to the bottom of the gel from the sample collected 4 weeks postpartum in comparison to the samples collected at 2 weeks gestation to 6 weeks prepartum. For the purpose of discussion, the increased Coomassie-staining substance or substances in the electrophoretic zone indicated in Fig. 1 will be subsequently designated as pregnancy-associated protein. Figure 2 further demonstrates the 4 week postpartum increase in pregnancy-associated protein by comparison of the postpartum pattern of a second individual with control subjects. No difference was observed between male donors and female donors not pregnant within the previous 12 months in Coomassie blue staining within the electrophoretic zone which contains the pregnancy-associated protein. Pregnancy-associated protein also was detected 4 to 6 weeks postpartum from the other six subjects who were studied throughout pregnancy and postpartum.

Electrophoresis also demonstrated increased staining in the electrophoretic zone which contains pregnancy-associated protein. Quantitation of the amount of protein in the zone, however, was found impractical because of incomplete resolution of the multiple bands present on the gels, the large variation in concentration of the various proteins present in the gels, and the unavailability of pregnancy-associated protein for quantitative calibration.

Minimal, if any, pregnancy-associated protein was present in any subject prior to birth before term. A high concentration of pregnancy-associated protein in the electrophoretic zone was evident in all samples collected from one subject on each of the 9 days before delivery and at 1, 2, and 3 days postpartum (Fig. 3). Staining within the zone which contains pregnancy-associated protein was as intense as observed with this subject has been seen in only three of 400 parotid saliva samples from nonpregnant subjects analyzed by the present technique. The amount of pregnancy-associated protein present at these times,

however, was less than observed 4–6 weeks postpartum with the other subjects. The parotid salivary samples collected from the 55 donors 1–4 days postpartum also contained less pregnancy-associated protein than at 4–6 weeks postpartum. No pregnancy-associated protein was evident 4 months postpartum in the three subjects investigated.

Differences in the parotid protein patterns of samples collected at different times during pregnancy and postpartum, besides those noted above, are evident in Figs. 1 and 3. None of these other differences, however, appeared to be consistent among subjects. Samples must be collected at more frequent intervals and at carefully controlled time periods to define the potential relationship of these additional differences to pregnancy. The variation among gel patterns of parotid salivary samples collected from a subject throughout pregnancy was much greater than that among multiple samples collected from individual nonpregnant subjects at different times (5–7).

Discussion. The data in this paper show a marked increase in or appearance of an additional protein or proteins in a particular electrophoretic zone from human parotid saliva collected during late pregnancy, shortly after term, and 4–6 weeks postpartum. Careful examination of Figs. 1 and 2 suggests an apparent increase in staining intensity in more than a single band within the pregnancy-associated protein zone from samples collected 4–6 weeks postpartum. The specific event during pregnancy or postpartum with which the protein change is associated is not clear. However, since the amount of pregnancy-associated protein present in parotid saliva is much greater 4–6 weeks postpartum than either before term or immediately postpartum, it appears that the salivary change we have noted is more closely associated with postpartum processes, perhaps activation of lactation, than with pregnancy per se. Several hundred parotid salivary protein samples have been analyzed by the procedure used in the present study. The only other subject in whom a protein change comparable to that observed in 4–6 weeks postpartum subjects was a 16-year-old male with severe acne. Parotid salivary differences other than the pregnancy-

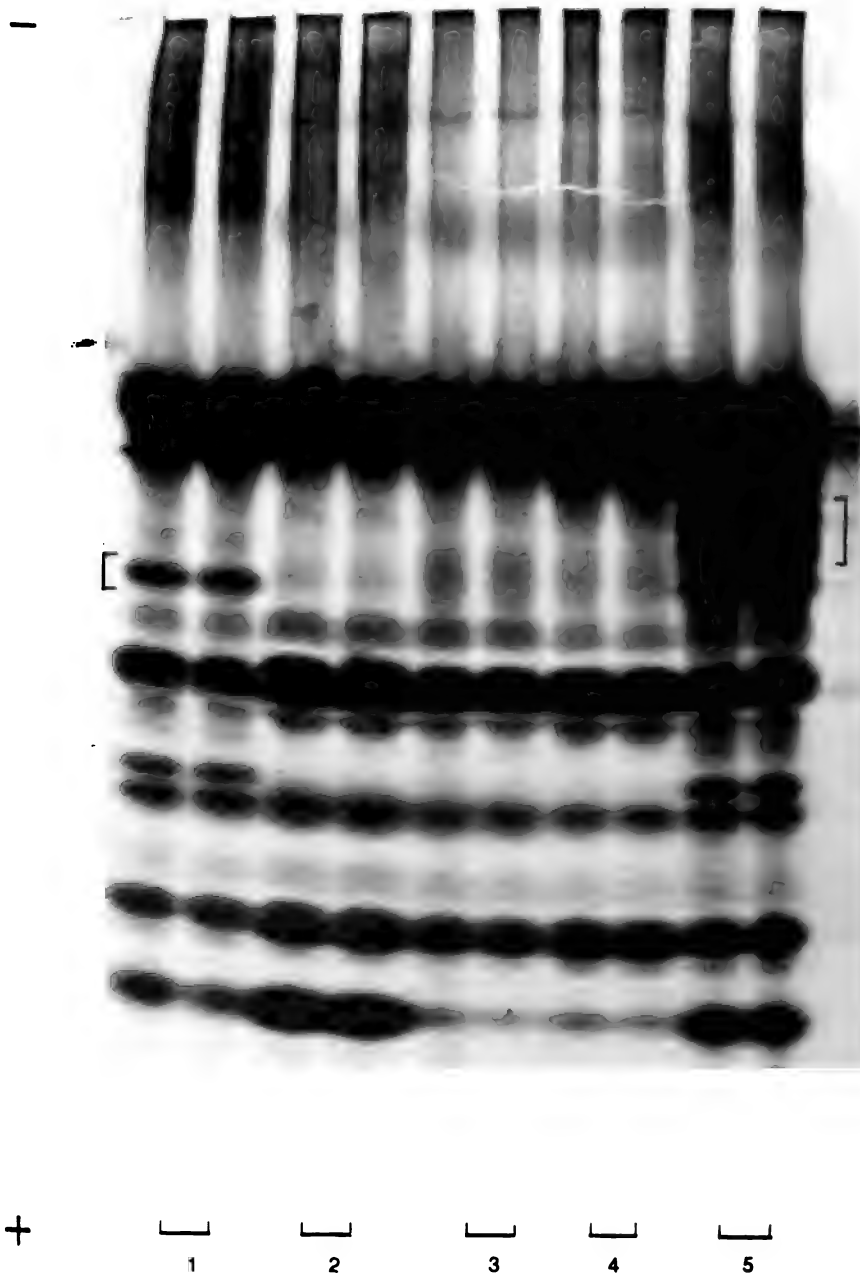


FIG. 2. Polyacrylamide gel slab patterns of the parotid salivary proteins of a 4 week postpartum sample (sample 5) compared with four control donors (samples 1-4). The bracket on the right margin of the figure indicates the electrophoretic zone which contains pregnancy-associated protein. The prominent staining area in sample 1 indicated by the bracket is not a pregnancy-associated protein and has an electrophoretic mobility slightly greater than the pregnancy-associated protein. This latter protein, also seen in sample 5 just below the pregnancy-associated protein, frequently occurs in high concentration in parotid saliva. Anode (+) and cathode (-) are indicated on the left margin of the gel.

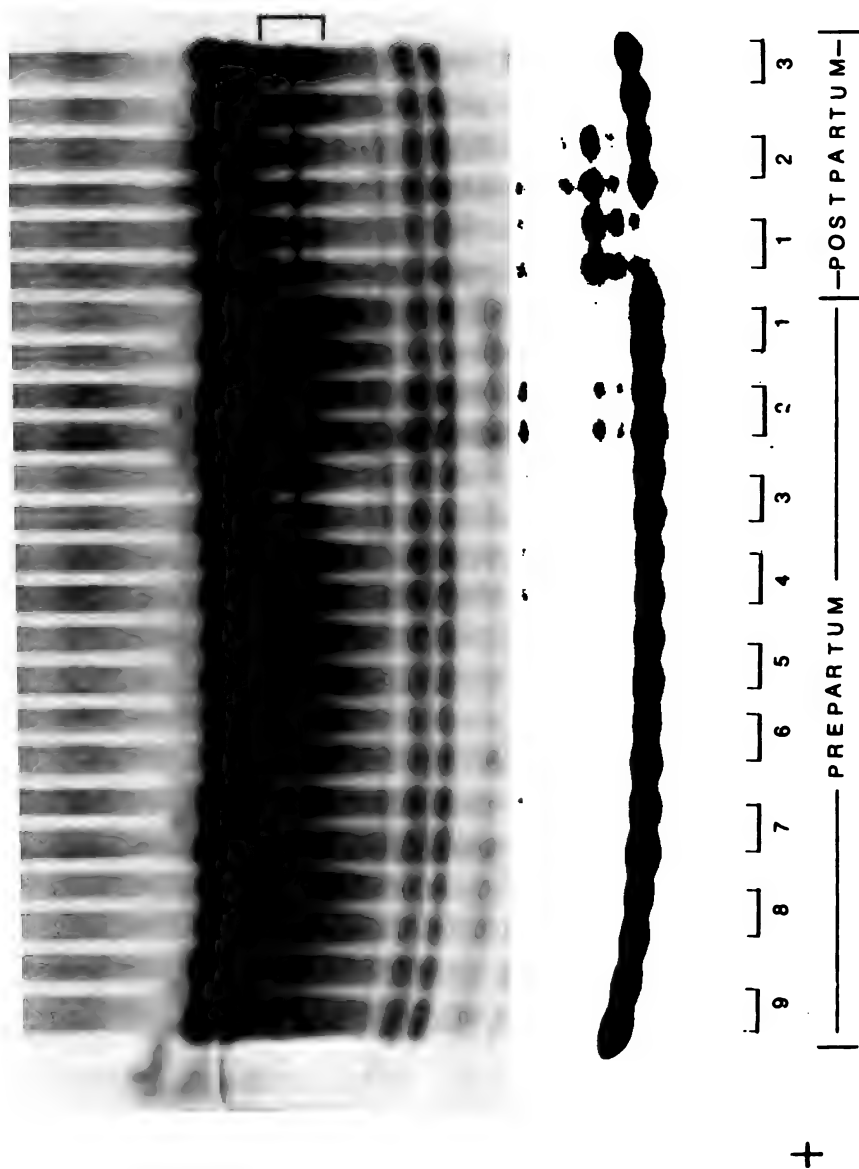


FIG. 3. Polyacrylamide gel slab patterns of parotid salivary proteins collected from one subject on each of the 9 days prior to delivery and 1, 2, and 3 days postpartum. Time of sample collection (days pre- or postpartum) is indicated by the numbers on the bottom of the figure. The electrophoretic zone containing pregnancy-associated protein is shown by the bracket on the right margin of the figure. Anode (+) and cathode (-) are indicated on the left margin of the gel.

associated protein were evident in some but not all subjects during pregnancy and postpartum. These additional changes, although not equivalent in all subjects, indicate that multiple changes are likely to occur in salivary proteins during pregnancy and postpartum.

The biochemical nature of the pregnancy-associated protein has not been identified yet. It occurs in an area of the gel in which small amounts of protein are obtained from parotid saliva of most control subjects. Thus, the pregnancy-associated protein may be a large increase in secretion of a normal salivary protein or proteins. Characterization studies performed in our laboratory and electrophoretic mobility indicate that the pregnancy-associated protein is probably not one of the several given in Table I. Amylase, the prominent staining area at the interface between the 6 and 10% gels, has a lower electrophoretic mobility than the pregnancy-associated protein. Immunoglobulins IgA and IgG are restricted to the 6% portion of the gel. The protein is not strongly bound by hydroxyapatite, nor does it contain unusually large quantities of proline. The pH of the gel system is such that proteins with very basic isoelectric points, such as lysozyme and small molecular weight basic proteins, are excluded from the gel. Albumin and transferrin have been demonstrated by crossed immunoelectrophoresis to have greater electrophoretic mobilities than the pregnancy-associated protein. Recent studies in our laboratory with

purified proteins show that lactoferrin and lactoperoxidase also have electrophoretic mobilities less than the pregnancy-associated protein. The pregnancy-associated protein has an electrophoretic mobility similar to acid and alkaline phosphatases, substances with molecular weights of 80,000-100,000. However, no other data are presently available to indicate that the pregnancy-associated protein is either of these enzymes. Periodic acid-Schiff stains of the gels suggest that the pregnancy-associated protein may contain a significant amount of carbohydrate.

Summary. Stimulated parotid saliva was collected from: (i) eight pregnant subjects at intervals from about 2-3 months gestation to 2 weeks prepartum and at 4-6 weeks postpartum; (ii) three of the above eight donors 4 months postpartum; (iii) one individual each of the 9 days prior to delivery and at 1, 2, and 3 days postpartum; (iv) 55 individuals 1-4 days postpartum; (v) 12 male and 12 female control subjects. A marked increase in or appearance of an additional protein or proteins was detected in a particular electrophoretic zone from the saliva collected during late pregnancy, shortly after term, and 4-6 weeks postpartum. The amount of this yet unidentified protein or proteins is greater in parotid saliva collected 4-6 weeks postpartum than obtained either immediately pre- or postpartum.

TABLE I. SALIVARY PROTEINS TENTATIVELY EXCLUDED AS THE PREGNANCY-ASSOCIATED PROTEIN.

1. Amylase
2. IgA, IgG
3. Proline rich proteins
4. Proteins strongly bound by hydroxyapatite
5. Small molecular weight highly basic proteins
6. Lysozyme
7. Serum albumin
8. Transferrin
9. Lactoferrin
10. Lactoperoxidase

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Binding Sites of Bromoacetylcholine in the Rat Diaphragm¹ (39520)

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acetylcholine, a halogenated ana-
etylcholine, has been shown to pro-
linergic effects similar to acetylcho-
2). It has also been reported that
etylcholine binds irreversibly to
ic receptors at nicotinic sites (3, 4).
re the possibility of the use of bro-
choline as a tagging agent for the
of cholinergic receptors, the bind-
of this compound in the rat dia-
were studied. Since both bromoace-
: and its hydrolytic product, bro-
e, may act as alkylating agent and
oncholinergic sites, the binding of
state in the rat diaphragm was also

als and methods. Materials. Bromo-
tylcholine perchlorate (¹⁴C-BrACh)
hesized according to the method
l previously (5). Bromo-[1-¹⁴C]-
mide (specific activity: 1.1 mCi/
New England Nuclear) was reacted
ine bromide to yield bromoacetyl-
romide which was then converted
chlorate salt with 70% perchloric
in absolute ethanol medium. The
Ch synthesized had a specific ac-
1.0 mCi/mmole. Bromo-[1-¹⁴C]-
id (¹⁴C-BrAcet) with a specific
of 1.23 mCi/mmole was obtained
w England Nuclear. [³H]Nicotine
pecific activity of 250 mCi/mmole
ethyl-[³H]acetylcholine with a spe-
ivity of 250 mCi/mmole were ob-
om Amersham/Searle.

ls. Holtzman rats were anesthe-
1 ether. Their diaphragms were re-
id weighed. The diaphragms were
ito three groups and each group of
ns was suspended in 5 vol of 100

mM phosphate buffer (pH 7.4) contain-
ing 0.95 μ M diisopropylfluorophosphate
(DFP). In group one, 0.2 mM (final concen-
tration) of ¹⁴C-BrACh was added. In group
two, 0.2 mM ¹⁴C-BrAcet was added. In
group 3, 0.2 mM ¹⁴C-BrACh and 0.2 mM
bromoacetic acid (unlabeled) were added.
The diaphragms were then incubated in
these three media for 30 min at 25°C. Each
group of diaphragms was then taken out of
the incubation medium and resuspended in
phosphate buffer to wash out the excess ¹⁴C-
BrACh or ¹⁴C-BrAcet. The washing process
was repeated two more times. The washed
diaphragms from each group were then sus-
pended in 5 vol of sucrose (250 mM)-phos-
phate (100 mM) buffer (pH 7.4) containing
0.95 μ M DFP, minced, and homogenized
with a Polytron (Brinkman Instruments) ho-
mogenizer. The homogenate from each
group was centrifuged at 100,000g for 90
min at 4°. The pellet was resuspended in 5
vol of phosphate buffer and centrifuged
again at 100,000g for 90 min. The process
was repeated one more time to remove all
the unbound ¹⁴C-BrACh or ¹⁴C-BrAcet.
The pellet so obtained in each group was
resuspended in 2 vol of phosphate buffer
containing 1.5% Triton X-100 and kept
overnight at 4°. It was centrifuged at
100,000g for 90 min at 4°C. The residue
was discarded and the supernatant fractions
containing ¹⁴C-BrACh or ¹⁴C-BrAcet and
the solubilized membrane proteins were fur-
ther purified for ¹⁴C-BrACh-binding pro-
teins by column chromatography.

The supernatant fractions containing ¹⁴C-
BrACh or ¹⁴C-BrAcet and solubilized mem-
brane proteins were passed through a Seph-
adex G-200 column (2.5 × 40 cm) pre-
equilibrated with phosphate buffer contain-
ing 1.5% Triton X-100. The column was
eluted with the same buffer and 1- to 3-ml
fractions were collected using a fraction col-
lector. The protein concentration in each

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17854) awarded by the National Cancer
Department of Health, Education and Wel-

fraction was determined by the method of Lowry *et al.* (6) with the modification that the white precipitates of Triton X-100 were removed by centrifugation before reading the blue color of the reaction. The validity of this method was confirmed by performing the determination on a known amount of albumin with and without Triton X-100 and also comparing this method with that of Wang and Smith (7). The radioactivity of each fraction was determined in a liquid scintillation counter.

In a fourth group of experiments, the ^{14}C -BrACh-binding proteins were isolated from rat diaphragms using the homogenization, centrifugation, and column chromatography on Sephadex G-200 procedures outlined above but without using ^{14}C -BrACh or ^{14}C -BrAcet as a tagging agent. The binding of $[^3\text{H}]$ nicotine and $[^3\text{H}]$ acetylcholine in this fraction was then studied by equilibrium dialysis. The inner compartment consisted of a bag of dialysis tubing containing 3.0 ml of binding protein solution (1 mg of protein/ml). The outer compartment contained 1000 ml of phosphate buffer with $[^3\text{H}]$ nicotine ($1.2 \times 10^{-9} \text{ M}$) or $[^3\text{H}]$ acetylcholine ($5.4 \times 10^{-10} \text{ M}$) in a stoppered Erlenmeyer flask. The dialysis was carried out in a cold room at 4° for 24 hr with constant stirring. Samples were taken from the dialysis bag and the outer compartment, and the radioactivity was counted.

Results. Figure 1 shows an elution profile of the rat diaphragm fraction containing solubilized membrane proteins and ^{14}C -BrACh when passed through a Sephadex G-200 column. It shows one peak of radioactivity (^{14}C -BrACh) and a parallel protein peak coming out immediately after the void volume. The molar concentration of ^{14}C -BrACh in fraction No. 34 (peak of radioactivity) was 3.3 nmole/ml as calculated from the specific activity. The protein concentration in this fraction was 1.9 mg/ml. Assuming that ^{14}C -BrACh binds mole/mole to the receptor protein, the concentration of ^{14}C -BrACh-binding protein would be 1.7 nmole/mg of total proteins in this fraction.

Figure 2 shows an elution profile of the rat diaphragm fraction containing solubilized membrane protein and ^{14}C -BrAcet when passed through a Sephadex G-200 col-

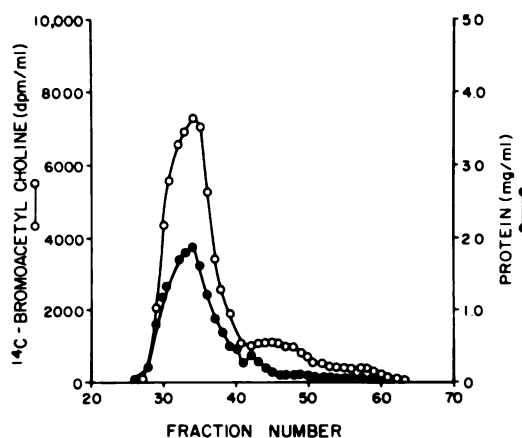
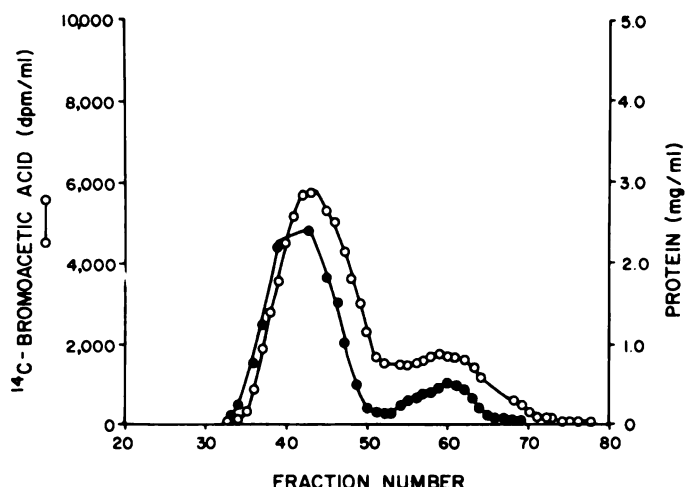


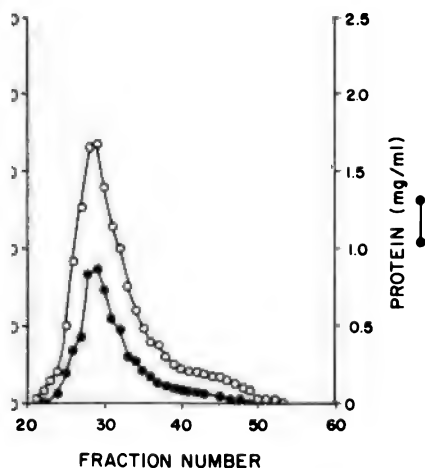
FIG. 1. Elution profile of ^{14}C -BrACh-treated rat diaphragm tissue on Sephadex G-200. The column was equilibrated with 100 mM phosphate buffer, pH 7.4, containing 1.5% Triton X-100. Elution was done with the same buffer. (●—●) Protein concentration; (○—○) ^{14}C -BrACh.

umn. It shows one large and another small peak of radioactivity with parallel protein peaks. The first peak of radioactivity and protein was reached in fraction No. 43 because of the collection of smaller fractions as compared to the ^{14}C -BrACh column. The molar concentration of ^{14}C -BrAcet in fraction No. 43 was 2.1 nmole/ml as calculated from the specific activity. The protein concentration in this fraction was 2.4 mg/ml. Assuming that ^{14}C -BrAcet binds mole/mole to the receptor protein, the concentration of ^{14}C -BrAcet-binding protein would be 0.88 nmole/mg of total proteins in this fraction.

Figure 3 shows the elution profile of the rat diaphragm fraction containing solubilized membrane protein and ^{14}C -BrACh and bromoacetic acid (unlabeled) when passed through a Sephadex G-200 column. It shows one radioactivity peak of ^{14}C -BrACh and a parallel protein peak. The molar concentration of ^{14}C -BrACh in fraction No. 29 (peak of radioactivity) was 1.7 nmole/ml as calculated from the specific activity. The protein concentration in this fraction was 0.9 mg/ml. Assuming that ^{14}C -BrACh binds mole/mole to the receptor protein, the concentration of ^{14}C -BrACh-binding protein in the presence of bromoacetic acid would be 1.9 nmole/mg of total proteins. This value is not much different from the one obtained



Elution profile of ^{14}C -BrAcet-treated rat diaphragm tissue on Sephadex G-200. The column was equilibrated with 100 mM phosphate buffer, pH 7.4, containing 1.5% triton X-100. Elution was done with the same buffer. (●—●) Protein concentration; (O—O) ^{14}C -BrAcet.



Elution profile of ^{14}C -BrACh-treated (in the presence of unlabeled BrAcet) rat diaphragm tissue on Sephadex G-200. The column was equilibrated with 100 mM phosphate buffer, pH 7.4, containing 1.5% triton X-100. Elution was done with the same buffer. (●—●) Protein concentration; (O—O) ^{14}C -BrACh.

binding of ^{14}C -BrACh in the absence of bromoacetic acid, indicating that bromoacetic acid does not bind to bromoacetic acid binding sites.

Figure 1 shows the binding of $[^3\text{H}]$ nicotine to proteins of peak I (Fig. 1) when eluted without the tagging agent. The total protein concentration in the pooled frac-

tion was 1.0 mg/ml. It is clear that there was a net increase of $[^3\text{H}]$ nicotine inside the dialysis bag due to the binding of nicotine to the solubilized membrane proteins. Results obtained with $[^3\text{H}]$ ACh were similar to those obtained with $[^3\text{H}]$ nicotine (Table I).

Discussion. According to the results of the ^{14}C -BrACh-binding experiments, the solubilized fraction of rat diaphragms contained approximately 1.8 nmole of ^{14}C -BrACh-binding sites per milligram of total proteins. In our experiments 1% of rat diaphragm proteins was solubilized by Triton X-100 and collected in peak I of Sephadex G-200 column. Therefore, we estimate that the rat diaphragm has about 18 nmole of ^{14}C -BrACh-binding sites per gram of tissue. Our results indicate that ^{14}C -BrACh-binding sites are more than 1000-fold higher than that of α -bungarotoxin-binding sites which range from 1.3 to 4 pmole/g of rat diaphragm (8–12). The reason for more ^{14}C -BrACh-binding sites as compared to α -bungarotoxin is not known. One of the possible reasons would be that BrACh binds to non-specific sites via alkylation just as BrAcet does. However, this possibility was ruled out because the binding of ^{14}C -BrACh was not affected by the combined treatment of the tissue with ^{14}C -BrACh plus cold BrAcet. Although BrACh produces biological effects of the cholinergic type which can be

TABLE I. BINDING OF [³H]NICOTINE AND [³H]ACETYLCHOLINE TO SOLUBILIZED CHOLINERGIC RECEPTOR PROTEIN.^a

Compound	Free compound added into dialysis medium (A)		Free compound plus compound bound to receptor proteins inside the dialysis bag (B) (dpm/ml)	Net amount of compound bound to receptor protein [(B)-(A)]	
	(dpm/ml)	(nM)		(dpm/ml)	(nM)
[³ H]Nicotine	790 ± 50	1.2 ± 0.2	1180 ± 70	390 ± 22	0.69 ± 0.1
[³ H]Acetylcholine	300 ± 20	0.54 ± 0.1	664 ± 40	364 ± 22	0.66 ± 0.1

^a The values given here are means ± SE of three different experiments.

blocked by cholinergic and cholinolytic agents (1-4), it is still possible that BrACh binds to proteins other than cholinergic receptors. On the other hand, it is also possible that the large molecule of α -bungarotoxin which binds to one of the cholinergic receptors also covers up the adjacent cholinergic receptors just like an umbrella (4) which tends to give a lower number of cholinergic binding sites.

Summary. To explore the possibility of using bromoacetylcholine as a tagging agent for the isolation of cholinergic receptors, the binding sites of this compound in the rat diaphragm were studied in the presence and absence of bromoacetate. The solubilized membrane fraction of rat diaphragm was found to contain 18 nmole of ¹⁴C-BrACh-binding sites per gram of tissue. The presence of bromoacetate did not change the binding of ¹⁴C-BrACh. The estimated binding sites of ¹⁴C-BrACh were 1000-fold higher than those of α -bungarotoxin binding sites.

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Carbohydrate Energy Sources for Chinese Hamster Cells in Culture¹ (39521)

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Mammalian cells in culture are supposed to use as a source of energy (1) and it is not clear how many other carbohydrates support their growth. With the view of clarification of the situation and perhaps providing for development of additional genetic systems by which to characterize cells in culture, we have tested a number of substrates for their ability to replace glucose in the culture medium.

Materials and methods. 1. *Cell lines and culture conditions.* Three Chinese hamster cell lines were studied: strain CHO-K1 from hamster ovary (2), strain V79 from hamster lung (3), and strain CHS from Chinese hamster embryo (ERICR).

For the maintenance of these cultures in serum, the medium was supplemented with fetal calf serum as described previously (4, 5).

Source of the carbohydrates. The carbohydrates tested were of the highest purity available and were obtained from either Sigma Chemical Laboratories (Waukegan, Ill.) or Chemical Co. (St. Louis, Mo.).

Enzyme assays. To test the ability of the cell lines to grow on various carbohydrates, the carbohydrates were substituted for glucose in F12 at an equal concentration ($2 \times 10^{-2} M$) or, in the case of glycogen, at an equal weight. The serum supplement was a 1:10 molecular portion of fetal calf serum (FCS) prepared by passing serum over a Sephadex G-50 column (6). FCS was added to 7% of the total volume. The cells were seeded and permitted to attach to the plates before the addition of the carbohydrates. The medium used for washing and other

manipulations of the cells was saline G (6) with glucose omitted. This is essentially a phosphate-buffered saline containing calcium and magnesium. The inoculum in the growth experiments was 200 cells/30-mm plate.

The cultural conditions for measurement of enzyme activity were to inoculate 5×10^5 cells/60-mm-diameter plastic petri dish. After 24 h of growth the cultures were dense but not confluent with many cells in mitosis. Cells were harvested with trypsin, centrifuged, and washed twice with an equal volume of G-saline without glucose, and finally resuspended at 10^7 cells/ml in 0.01 M phosphate buffer at pH 7.2. The cells were lysed by three cycles of freeze-thawing.

The ability of cell extracts of CHO-K1 to hydrolyze *p*-nitrophenyl derivatives of various carbohydrates was measured by adding extract to give 0.1 mg of protein to a 1-ml reaction mixture containing 0.1 M acetate buffer, pH 4.5, and 0.01 M *p*-nitrophenyl derivative. Incubation was at 37° for 15 hr. The reaction was terminated by the addition of 2 ml of 0.23 M Na_2CO_3 , the solution clarified by centrifugation, and the absorbance measured at 415 nm.

Results and discussion. The results, stated as a plating efficiency (EOP), which is the number of cells forming colonies (greater than 50 cells per colony) divided by the number of cells plated, are given in Table I. There are only a few carbohydrates which permit colony formation for each of the strains, and there are differences between hamster strains. It should be noted that the cells were not permitted to attach prior to the addition of the carbohydrates, and, if the substances did not permit or if they inhibited attachment, these experiments would not detect the effect. That attachment is important is indicated by the fact that cells of strain CHO-K1 allowed to at-

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tach in the presence of glucose and then exposed to trehalose give the appearance of growth (colony formation), whereas cells given continuous trehalose exposure show no signs of colony formation. The basis of this phenomenon remains obscure.

There are no disaccharides which serve as energy sources. This is surprising since CHO-K1 cell extracts possess enzymes for hydrolyzing a number of *p*-nitrophenyl derivatives of some of the carbohydrates at pH 4.5 (Table II). Presumably these enzymes are

TABLE I. CARBOHYDRATES TESTED AS ENERGY SOURCES FOR CHINESE HAMSTER CELLS

A. Carbohydrates not serving as energy sources (EOP < 0.01)

Strain CHO-K1	L-Arabinose, cellobiose, dulcitol, erythritol, α -L-fucose, α -D-fucose, galactolactone, galactose 6-phosphate, galacturonic acid, glucose-6-phosphate, glucuronic acid, α -methylglucoside, DL- α -glycerophosphate, lactose, guanosine diphosphate mannose, α -methylmannoside, mannitol, mannitol 1-phosphate, mannosamine, acetylmannosamine, melibiose, pyruvate, α -L-rhamnose, raffinose, salicin, sucrose, trehalose, turanose, D-xylose, D-arabinose, D-lyxose
Strain V79	L-Arabinose, cellobiose, dulcitol, erythritol, esculin α -L-fucose, α -D-fucose, inulin, lactose, mannose, melibiose, melizitose, α -L-rhamnose, raffinose, trehalose, turanose, D-lyxose
Strain CHS	Arabinose, fructose, galactose, lactose, mannose, melibiose

B. Carbohydrates serving as energy sources (EOP > 0.5)

Strain CHO-K1	Glucose, mannose (14- to 16-hr generation time), glucose-1-phosphate (20- to 24-hr generation time), maltose, ^a glycogen, ^a galactose (14- to 30-hr generation time), fructose (30- to 36-hr generation time)
Strain V79	Glucose (12- to 14-hr generation time), galactose (20- to 24-hr generation time), fructose (20- to 24-hr generation time), maltose ^a
Strain CHS	Glucose (16-hr generation time)

^a An artifact, since the serum in the medium hydrolyzes maltose and glycogen to glucose.

TABLE II. HYDROLYSIS OF *p*-NITROPHENYL DERIVATIVES OF A VARIETY OF CARBOHYDRATES BY EXTRACTS OF CHINESE HAMSTER OVARY CELLS.^a

<i>p</i> -Nitrophenyl derivative of	<i>p</i> -Nitrophenol (mM/0.1 mg of cell protein \times 18 hr)
α -Glucoside	10.27
β -Glucoside	10.04
α -Galactoside	48.00
β -Galactoside	47.04
α -Mannoside	19.18
β -Mannoside	0.00
α -L-Fucoside	44.16
β -D-Fucoside	1.71
β -D-Xylopyranoside	0.60
β -Glucuronide	48.00

^a pH = 4.5.

lysosomal in nature and used for the degradation of polysaccharides synthesized by the cells and cannot be used to provide energy for cell growth.

An effect of sugars on fibroblast morphology has recently been reported (7) but we have not observed any changes in morphology induced by sugars on the hamster lines studied here.

It should be noted that at least some batches of serum contain enzymes that degrade maltose and glycogen to glucose and which give the appearance of growth support. A similar observation has been made for starch as well as maltose (8). Fetal calf serum also appears to hydrolyze *p*-nitrophenyl- α -mannoside, but it is not known whether this is the same or a different enzyme.

Summary. Three Chinese hamster cell lines (CHO-K1, V79, and CHS) were tested to see which carbohydrates would support their growth in culture. There were differences between lines (mannose supported growth of CHO-K1 only), and glucose was the only carbohydrate that supported growth of all three strains. CHO-K1 cells possess enzymes for hydrolyzing some disaccharides which do not support growth. Fetal calf serum contains enzymes that hydrolyze maltose, glycogen, and α -mannosides.

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Plasma Prolactin and Progesterone during the Estrous Cycle in the Mouse¹ (39522)

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The estrous cycle of laboratory mice is influenced by the presence or absence of a male (1-3). Absence of a male results in irregular cycles when the females are individually caged, prolonged diestrus and pseudopregnancies when they are caged in small groups, and anestrus when they are caged in large groups. Females caged in the olfactory presence of a male exhibit shorter and more regular cycles and can be made to cycle synchronously by previous grouping in large numbers.

In all species thus far studied, including mice (3, 4), ovulation is preceded and presumably caused by a sharp rise in circulating LH and FSH. The ultimate regulation of ovulation, however, resides in an interaction between the pituitary and gonadal hormones (5). The interactions between pituitary and gonadal hormones during the estrous cycle have been extensively studied in rats (5-8). Hormone patterns during the estrous cycle in laboratory mice have not been as thoroughly investigated, despite the importance of mice in cancer and genetic studies. In the present study, we report the simultaneous measurement of both plasma progesterone and prolactin in the same strain of mice in which we previously characterized LH and FSH (3).

Materials and methods. Animals. Mice of a random-bred stock derived from a four-way cross, Line C (9), were maintained on a light schedule of 14 hr light (on between 0500 and 1900 hr). Nulliparous females, 60-70 days of age were synchronized (10) by being grouped 15 to a cage for 2 weeks then examined for vaginal smear pattern. Females showing evidence of cycling, i.e., a nucleated and/or cornified cell pattern, were eliminated from the study. Male and female animals were housed in separate cages (7 × 8 × 10.5 in.) which were com-

posed of wire mesh on three sides. The cages were placed so that a common mesh side of one cage was in contact with the other to allow olfactory and visual stimuli. Three females were placed in one cage and a mature male in the other. Vaginal smears were taken daily by saline lavage and examined in unstained wet preparations. The following criteria were used for identification of cycle stages: proestrus, nucleated or nucleated and cornified cells; estrus, cornified cells; metestrus, leukocytes and cornified cells; diestrus, leukocytes or leukocytes and nucleated cells. Only those females exhibiting two consecutive, 4-day cycles were used in the study. Females were sacrificed at intervals throughout their third or subsequent cycle within 10 sec after removal from their cages. The animals were killed by decapitation, their blood collected through heparinized funnels, and the plasma obtained following centrifugation stored frozen until assay.

Assays. Materials and protocol for the radioimmunoassay of prolactin were supplied by Dr. Y. N. Sinha (11). The prolactin concentrations are expressed as nanograms of the standard per milliliter of plasma. The biological potency of the mouse prolactin standard is 25 IU/mg. All prolactin samples were run in one assay. Plasma progesterone concentrations were determined by radioimmunoassay using an antiserum (provided by Dr. G. D. Niswender) produced in rabbits against progesterone conjugated to bovine serum albumin at the 6 position. The specificity of this antiserum is such that progesterone measurements can be made without prior chromatography (12). Water blanks were always undetectable and recoveries were between 60 and 85%. Low blood yield per animal necessitated the assay of progesterone at each time interval from a single plasma pool. To assure that each animal made an equal contribution to the pool, pools were made up of 10 μ l from each

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il (8 to 12 animals/pool). All progesterone samples were included in one assay. In-assay coefficients of variation were 10.4% for prolactin and 7.4% for progesterone. The lowest detectable concentration was 10 ng/ml for prolactin and 0.8 ng/ml for progesterone. Times are based upon a 24-hr cycle.

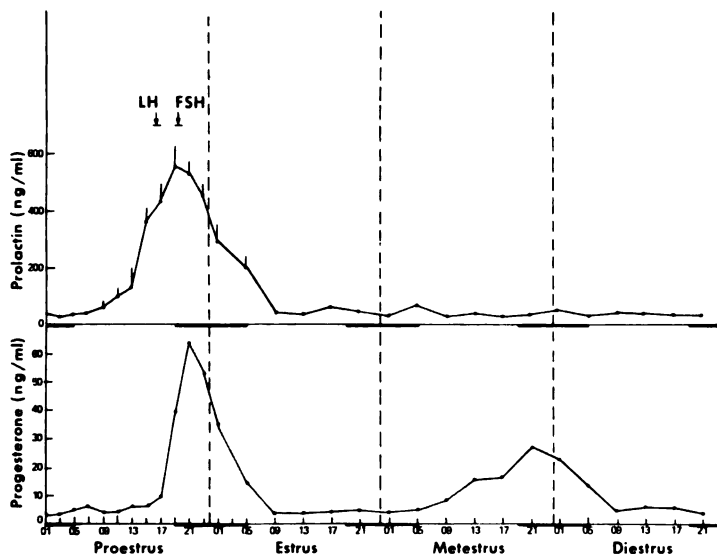
Statistical comparisons of peak concentration to baseline levels (sum of all baseline values) were determined by a confidence interval of the mean.

Results. The concentrations of prolactin and progesterone in plasma of Line C females during different stages of the estrous cycle are shown in Fig. 1. Concentrations of prolactin were around 50 ng/ml throughout the estrous cycle except during proestrus. 100 hr on the morning of proestrus, elevated levels were found (93 ± 32 ng/ml). During the afternoon of proestrus, prolactin rose to a peak concentration of 64 ng/ml at 1900 hr. This increase was significant ($P < 0.01$) over baseline values. After 1900 hr, the prolactin levels began to decrease and by 0900 hr on the day

of estrus, the concentrations were back to around 50 ng/ml.

The pattern of progesterone secretion during the cycle consisted of two major surges, one during the late afternoon of proestrus and the other on the morning of metestrus. Progesterone values remained low (around 5 ng/ml) until the afternoon of proestrus when a surge occurred, reaching a peak concentration of 64 ng/ml at 2100 hr. After this time, a precipitous drop in progesterone levels occurred, falling to around 5 ng/ml by 0900 hr on the morning of estrus. The second surge occurred during metestrus reaching a peak level of 27 ng/ml at 2100 hr. Both surges were significant changes from baseline concentrations ($P < 0.01$). Elevated levels were followed by a decline to baseline values of around 5 ng/ml by 0900 hr on diestrus.

Discussion. The pattern of progesterone secretion during the estrous cycle described here for mice is similar to that of the rat (7, 8). Both species show two major increases, during metestrus and on the afternoon of proestrus, and progesterone levels peak



1. The pattern of prolactin and progesterone during the mouse estrous cycle. Eight to 12 animals were bled at each time interval and the plasma subjected to the two hormone analyses. Prolactin was determined in individual animals. Standard errors are not shown on some prolactin means because they are smaller than the size of the dot on the graph. At each time interval, progesterone was determined in a single plasma pool, hence no standard errors are indicated. The numbers along the abscissa represent the time of day (24-hr clock). Dark bars represent the dark period (1900-0500 hr) and the dashed lines denote midnight. The proestrus surge of LH and FSH secretion are indicated by arrows (\downarrow) (data taken from ref. (3)).

after initiation of the proestrous surge of prolactin. Some investigators (13-15) report that in rats serum progesterone levels display a circadian rhythm during the estrous cycle. If mice have a similar diurnal rhythmicity of plasma progesterone levels, it could have been missed since samples were collected only at 4-hr intervals at stages other than proestrus.

In an earlier study (16) of plasma progesterone levels during pregnancy and parturition in Line C mice, we found that levels of progesterone were as low as baseline levels of the estrous cycle (around 5 ng/ml) only on the day of parturition. From Days 2 through 9 of pregnancy, concentrations of progesterone were between 41 and 54 ng/ml. These values are about midway between the peak of 64 ng/ml found during proestrus and the peak of 27 ng/ml found during metestrus. The highest level of progesterone (113 ng/ml) was found on Day 15 of pregnancy.

The pattern of prolactin secretion during the estrous cycle of mice has been reported only in two preliminary studies, Yanai and Nagasawa (17) and Sinha *et al.* (18). In the former study, mice were maintained on the same light schedule as those in the present study, but they were sacrificed at only a few time intervals. Yanai and Nagasawa found that the highest level of plasma prolactin (around 120 ng/ml) occurred on the late afternoon of diestrus. At all other stages of the cycle, prolactin levels were around 50 ng/ml, which is the same baseline level we found using the same RIA (11). Their failure to find elevated prolactin concentrations during the afternoon of proestrus was probably due to either sacrificing animals at only one time interval (1700-1730 hr) or due to a strain difference (they used strain C3H/He). The elevated levels of prolactin which they found on diestrus might possibly be due to bleeding the animals under ether anesthesia (19), although it is not clear why this stage of the cycle should be more susceptible to the stressful method of blood collection used by these investigators.

In the studies of Sinha *et al.* (18), the animal quarters were lighted from 0600 to 2000 hr daily. In the two strains of mice which they studied (C3H/St and C57BL/

St), levels of serum prolactin were higher during the afternoon of proestrus than at any other stage of the cycle with concentrations of 111 and 153 ng/ml at 1400 hr on proestrus. Since these investigators sampled animals at only one time interval during the afternoon of proestrus, these levels are probably not the peak concentrations.

Linkie and Niswender (20) found in the rat that, except for Day 0 of pregnancy (day of the vaginal plug) and the day prior to parturition, circulating prolactin levels were equivalent to or less than concentrations found during the diestrous phase of the cycle. In mice, however, the baseline values of around 50 ng/ml found throughout all stages of the cycle except for proestrus are generally lower than those which we previously reported in pregnant animals of Line C (21).

The times when peak levels of LH and FSH occurred in Line C mice on the same light schedule as in the present study are shown in Fig. 1 (3). In rats, the patterns of LH, FSH, and prolactin secretion are similar during most of the estrous cycle with concentrations remaining low until the afternoon and evening of proestrus (8). All three hormones in rats peaked at about the same time, around 1700 hr on proestrus (1 hr before the dark period). In mice, peak levels of FSH and prolactin occurred at the beginning of the dark period while peak levels of LH were found 2 and 3 hr earlier during the light period. In both rats and mice, prolactin levels began to increase from baseline levels during the morning of proestrus, before LH, FSH, and progesterone began to rise. A brief peak of prolactin on the afternoon of estrus in rats was reported by Butcher *et al.* (7) but was not found in mice or rats by Smith *et al.* (8).

There is some disagreement in the rat studies as to whether the proestrous surge of LH occurs before (22), simultaneous with (7, 8, 23), or after (24) the progesterone surge. The temporal relationship of LH and progesterone cannot be established with certainty in mice since the two hormones were measured in similar groups of animals but not in the same individuals. If the two groups can be considered equivalent then it would appear that in mice the proestrus rise

a LH slightly precedes that of progesterone.

and collaborators (5, 6, 8) have intensively studied and thoroughly discussed pituitary-ovarian interactions which regulate estrous cyclicity in rats. Although LH has been shown to be the stimulus for the estrous surge of progesterone, the role of progesterone surge has not as yet been established. The preovulatory prolactin release, as far as presently known, does not play a major regulatory role in the estrous cycle block in the surge does not disrupt estrus. The prolactin surge at proestrus does not, however, cause lysis of the corpora lutea from the previous cycle. Although the estrus is not exactly identical to the rat in all aspects of hormone secretion, the present study and that which we reported previously indicate a substantial amount of similarity; hence we conclude that the same mechanisms are probably operating.

Materials and Methods. The concentrations of prolactin and progesterone in plasma of Line C rats during different stages of the estrous cycle were measured by radioimmunoassay. In the afternoon of proestrus, prolactin peak concentration at 1900 hr. The surge of progesterone consisted of two surges, beginning during the late afternoon of proestrus and the morning of estrus.

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Latent *Herpesvirus hominis* from Trigeminal and Sacral Dorsal Root Ganglia of *Cebus* Monkeys¹ (39523)

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Herpesvirus hominis type 2 (HVH-2) genital infection constitutes a uniquely important public health problem. This agent not only causes morbidity during primary infection but variable proportions of its victims also suffer recurrent disease. So far, experimental studies of latency and reactivation utilized rabbits and mice, and it remains uncertain whether the results can be extrapolated to primates. *Cebus* monkeys infected by intravaginal instillation of HVH-2 develop an infection similar to that seen in humans (1). To date, however, no systematic effort has been made to identify latent HVH-2 in experimentally infected simians. In this paper we present preliminary results indicating that HVH-2 induces a latent infection in sacral dorsal root ganglia of vaginally infected *Cebus* monkeys.

Materials and Methods. *Virus.* We used *Herpesvirus hominis* type 2, strain 333 (HVH-333), kindly provided by Dr. W. E. Rawls, Baylor College of Medicine, Houston, Tex. HVH-333 was originally isolated from a human penile lesion. After ultraviolet irradiation it transformed hamster embryonic fibroblasts and produced tumors in weanling hamsters (2). We grew the virus in FT cells (3). A single lot, titering 1×10^6 PFU/ml, provided the animal inocula.

Monkey inoculation and sampling. This study used sexually mature *Cebus albifrons* monkeys. Prior to the experiment all monkeys lacked evidence of HVH infection, as determined by multiple attempts to isolate virus from the oral cavity and genital tract and by the absence of serum neutralizing antibodies.

We infected monkeys intravaginally by depositing 5×10^5 PFU of virus into the vaginal vault and then inserting either a cotton pledget or a gelatin sponge. Three animals also received ocular inoculations, by dropping virus onto the eye and gently rubbing with a swab. Monkeys were examined and sampled three times weekly during the first month postinoculation, then twice weekly.

Virus isolation. Vagino-cervical, pharyngeal, and ocular swabs were inoculated onto FT cells for virus isolation. Finely minced tissue obtained at necropsy was homogenized and explanted or cocultivated with FT cells. FT cells inoculated with 10% tissue homogenates were checked for cytopathic effects weekly for 5 weeks.

Upon forming confluent monolayers, explanted cells were subcultured for further experimentation. Since thymidine analogs can induce nonpermissive cells to produce Epstein-Barr virus (4, 5) and cytomegalovirus (6) as well as depress interferon synthesis (7), we attempted a series of experiments using iododeoxyuridine (IUDR). For 4 consecutive weeks media from one set of subcultures were passed to FT cells and to FT cells grown 3 days in IUDR (20 mg/ml). Additional sets were grown with IUDR or cocultivated with FT cells which had been grown for 3 days in IUDR. We examined these cultures over 4 consecutive weeks, passing their media to FT cells. A fourth set of subcultures was maintained 3 to 4 weeks at 33° without medium change, then medium was passed to FT cells and IUDR-treated FT cells.

Presumptive HVH isolates were specifically identified as HVH-2 by tube neutralization and the immunoperoxidase antibody technique (8) using guinea pig hyperimmune serum to HVH-333 and an HVH type 1 strain.

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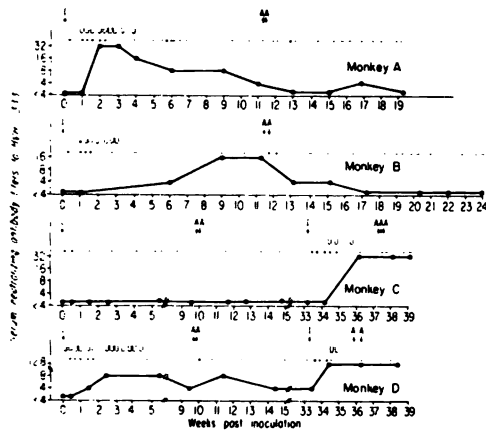


FIG. 1. Summary of clinical, serological, and virological patterns of monkeys A-D. ↓, HVH-333 inoculation; ▲, adrenalalin administered; ●, neutralizing antibody titer; +, HVH-333 genital isolate; -, genitalia negative for HVH-333; U, genital ulcer; V, genital vesicle.

Serology. We used a plaque reduction assay to measure neutralizing antibody to HVH-333 (9). Sera were diluted 1:4, inactivated 30 min in a 56° water bath and serially diluted. Diluted serum, 60–120 PFU of HVH-333 and 15 hemolytic units of guinea pig complement were incubated for 1 hr at room temperature prior to plating on FT cells. Serum titers were defined as the serum dilution producing at least 80% plaque reduction.

Results. We infected and killed five *Cebus* monkeys. Monkeys A and B were sacrificed at 23 and 19 weeks following genital infection. Figure 1 summarizes their clinical courses, viral excretion, and antibody patterns. Although both developed herpetic vulvovaginitis, seroconverted, and demonstrated spontaneous or epinephrine-induced shedding of HVH-2, we failed to isolate virus from their tissues (Table I).

TABLE 1. RECOVERY OF HVH-2 FROM *Cebus* MONKEY TISSUES.

Monkey Day killed Specimen	A 135			B 163			C 36			D 43			E 7		
	Ho- mog- enate	Co- cul- ture	Ex- plant	Ho- mog- enate	Co- cul- ture	Ex- plant	Ho- mog- enate	Co- cul- ture	Ex- plant	Ho- mog- enate	Co- cul- ture	Ex- plant	Ho- mog- enate	Co- cul- ture	Ex- plant
Pharyngeal swab	-			-			-			-			+		
Conjunctival swab							-			-			+		
Vagino-cervical swab	-			-			-			-			+		
Urine							-			-					
Vagina	-	-	-	-	- ^a	- ^d	-	-	- ^b	-	- ^a	-	+	+	+
Cervix	-	-	-	-	-	- ^d	-	-	- ^a	-	-	- ^d	+	+	+
Uterus		- ^a	- ^b	-	-	- ^d	-	-	- ^c	-	-	-	-	+	-
Ovaries	-	- ^a	-	-	-	- ^d	-	-	- ^b	-	-	- ^c	-	-	-
Bladder	-			-	-	- ^d	-	-	- ^b	-	-	- ^d	+	+	-
Kidneys							-	-	- ^b	-	-	- ^d	-	-	-
Inguinal nodes	-	-	-	-	- ^a	- ^b	-	-	- ^b	-	-	- ^d	-	-	-
Pelvic nodes	-	-	-	-	- ^a	- ^b	-	-	- ^b	-	-	- ^d	-	-	-
Paracervical fascia (ant)				-	-	- ^b	-	-	- ^c	-	-	- ^d	-	-	-
Paracervical fascia (post)	-	-	- ^c	-	-	-	-	- ^a	-	-	-	-	-	-	-
Liver	-			-	-	-	-	-	- ^a	-	-	- ^d	-	-	- ^a
Spleen	-			-	-	- ^d	-	-	- ^b	-	-	- ^d	-	- ^a	-
Spinal cord	-	-	-	-	-	-				-	-	- ^d	-	-	-
DRG L1, 2							-	-	-	-	-	- ^d	-	-	-
DRG L3, 4, 5, S1							-	-	-	-	-	- ^d	-	-	-
DRG S2, 3							-	-	-	-	+	- ^d	-	+	-
DRG C1, 2							-	-	-	-	-	- ^d	-	-	-
Tonsils	-	-	-	-	-	- ^d	-	-	-	-	-	- ^d	+	+	-
Parotid gland	-	- ^a	-	-	- ^a	- ^b	-	-	-	-	-	- ^d	-	- ^a	-
Left ophthalmic nerve							-	-	-	-	- ^a	-	-	+	-
Right ophthalmic nerve							-	-	-	-	-	- ^d	-	+	-
Left trigeminal ganglion							-	-	-	-	-	- ^d	-	-	+
Right trigeminal ganglion							-	-	-	-	-	- ^d	+	+	-

^a No tissue growth; however, media were passed weekly.

^b Media from explant passed to and explant cocultivated with IUDR-treated FT cells.

^c Same as ^b, in addition explant incubated at 33° and media passed to untreated and IUDR-treated FT cells.

^d Same as ^c in addition explant grown in IUDR.

^e Explant grown in IUDR.

^f Explant co-cultivated with IUDR-treated FT cells.

^g Same as ^e and ^f.

Monkeys C and D were reinoculated genitally and in the right eye 8 months after the primary infection. Sacrifice occurred 36 and 43 days later (Fig. 1). We isolated HVH-2 by cocultivation from pooled second and third sacral dorsal root ganglia (DRG) from monkey D, while all other tissues including lumbar, sacral, and coccygeal DRG were negative (Table I).

Monkey E was inoculated intravaginally and in both eyes with HVH-333. She developed herpetic conjunctivitis and exhibited vulvar herpetic lesions. When we killed her 7 days postinoculation the lesions remained HVH positive and she had yet to seroconvert. Several tissues yielded HVH-2 (Table I). The only positive tissues not directly contiguous with active infection sites were the ophthalmic nerves, trigeminal ganglia, and pooled second and third sacral DRG.

Discussion. Human HVH-2 genital infection has assumed increasing importance during recent years with respect to acute venereal disease, fetal and neonatal diseases, and cervical cancers. Our findings extend previous observations that *Cebus* HVH-2 infection closely resembles human genital disease clinically, virologically, and serologically (1). Thus *Cebus* should provide an excellent primate model to study the dynamics of HVH infection.

Our study also indicated that *Cebus* monkeys could serve as a model for examining viral latency in primates. Three of four animals manifested either spontaneous or epinephrine-induced recurrent viral shedding. Monkey D, killed 43 days postinfection, yielded HVH-2 isolates from pooled cocultivated second and third sacral DRG only. Other sacral, lumbar, and coccygeal DRG failed to yield HVH. HVH was not recovered from any tissues of monkeys A-C. In retrospect, however, we did not specifically dissect DRG from A or B. Although all tissues from monkey C remained HVH negative, she was the only animal not reshedding virus following epinephrine administration and may not have been latently infected. Monkey E was killed while still acutely infected. Virus was isolated from several tissues, all contiguous to actively infected areas. She also had HVH in two non-contiguous sites, the ophthalmic nerves, tri-

geminal ganglia and the second and third sacral DRG. These results are in accord with several mouse and rabbit studies. In these animals HVH appears to spread centripetally via intro-axonal transport and cause latent infection in neurons of sensory ganglia (10-13). Our results are also in accord with human cadaver studies reporting HVH isolations from cocultivated or explanted trigeminal ganglia (14-16) and sacral DRG (17). While human cadaver studies presume that virus in sacral DRG resulted from genital infections, only in mice has HVH been shown to spread from the vagina and cervix to lumbosacral ganglia and cause latent infection (13). Our study represents the first demonstration that genital HVH infection in primates results in viral latency limited to sensory ganglia specifically innervating the genitalia.

The findings from these five monkeys also indicated that latent HVH could only be recovered from sensory ganglia innervating the infected sites. We could not recover HVH from autonomic nervous tissue (paracervical fascia) nor from other tissues despite repeated attempts and a variety of techniques. Murine, rabbit, and human studies have not recovered latent HVH from other tissues (11, 15, 17-21). One previous study (22) examined tissue explants from *Cebus* monkeys following HVH-2 genital infection. They recovered HVH from explant cultures of vagina, uterus, bladder, ovary, adrenal, lung, kidney, and spleen from one to four of eight monkeys tested. This paper did not specify when postinoculation sacrifice occurred, nor did the authors state if neural tissue was examined. The five monkeys reported here represent our preliminary studies of *Cebus* genital HVH infection. Current experiments extend these preliminary observations and will examine the relationships of host age and immune response to genital HVH pathogenesis and latency.

Summary. Five *Cebus albifrons* monkeys were infected intravaginally with *Herpesvirus hominis* type 2. The resulting infection clinically, virologically, and serologically resembled that seen in humans. After the acute infection had ceased, one monkey shed virus spontaneously on two occasions

while two others shed following adrenalin administration. Herpesvirus was recovered from the second and third sacral dorsal root ganglia of two monkeys by cocultivation. Despite a variety of techniques, we could not recover virus from any other tissues. In addition, we recovered herpesvirus from cocultivated trigeminal ganglia of an ocularly infected monkey.

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Phagocytic and Bactericidal Activities of Pulmonary Macrophages following Sublethal Traumatic Shock¹ (39524)

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Phagocytosis of microorganisms and particulate matter by the alveolar macrophage is a pivotal mechanism utilized by the pulmonary reticuloendothelial system (RES) to inactivate pathogens invading the lower respiratory tract (1). The facilitation of phagocytosis by humoral opsonins as well as the postphagocytic stimulation of macrophage metabolism are well-known parameters of macrophage function and have recently been emphasized as to their importance in resistance of the lung to bacterial challenge. Several studies have demonstrated the existence and importance of opsonic antibodies found in respiratory secretions which are able to promote phagocytosis of *Pseudomonas aeruginosa*, a virulent lung pathogen in the compromised host (2, 3). Postphagocytic stimulation of respiration and glucose metabolism by lung macrophages has been linked to H₂O₂ production and peroxidative metabolism, thus delineating the formation of an effective bactericidal agent following bacterial ingestion by the alveolar macrophage (4). To what extent the phagocytic and metabolic activities of alveolar macrophages are altered acutely by injury has not been intensely investigated although of obvious importance in assessing pulmonary defense mechanisms against bacterial infection during a post-traumatic period.

The role of the systemic RES as a critical cellular defense mechanism in the host's response to shock and trauma has been intensely investigated (5-7). These studies have demonstrated that the macrophage population of the liver (i.e., Kupffer cell)

undergoes phasic changes following nonlethal shock or trauma manifested by early functional depression and subsequent RE recovery and stimulation. In contrast to this acute depression in hepatic phagocytic activity following injury, there appears to be an inverse response by the lung with increased pulmonary localization of blood-borne test colloids within 1 hr after the onset of trauma or injury (8, 9). The potential clinical importance of this pulmonary response to injury is further emphasized by the fact that a similar pattern exists following surgery (10) as well as during the course of metastatic spread following malignant tumor cell challenge (12). Thus, intravenous challenge of animals with viable tumor cells or test colloids during a period of postoperative Kupffer cell depression is manifested by delayed clearance from the blood and increased localization in the lung (10-12). The intent of the present study was to evaluate alveolar macrophage phagocytosis, serum opsonic responsiveness, and postphagocytic bactericidal activity following standardized whole-body sublethal trauma in order to define the status of the pulmonary macrophage system acutely after injury. A bacterial challenge of *Pseudomonas aeruginosa* was utilized to assess lung macrophage phagocytosis. The trauma model was a sublethal traumatic shock produced by Noble-Collip drum (NCD) trauma. This model was chosen due to its reproducibility and previous use to investigate systemic RES function *in vivo* (9).

Methods. Male Sprague-Dawley rats weighing 300-350 g were anesthetized by intraperitoneal injection of sodium pentobarbital (2 mg/100 g) and used in all studies. The Noble-Collip drum (NCD) trauma was performed at 40 rpm for 300 revolutions and all rats were anesthetized prior to trauma. This shock model resulted in less

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% mortality and has been previously reported (7, 9) as a sublethal trauma model. Alveolar macrophages (AM) were harvested prior to and following trauma by a modification of the method described by Myrvik (13). Inferior vena cava was used for serum collection and macrophages were obtained by repeated lung lavage with 0.2% EDTA in $\frac{1}{2}$ saline, pH 7.4, at 37° via the extratrachea. Cell pellets were washed in Hanks' balanced salt solution without glucose (HBSS) at a pH of 7.4 and at 4°. Differential counts were obtained by Wright-Giemsa staining techniques, and cell viability was determined by the exclusion of 1% trypan blue. Total cell counts were performed in duplicate by routine hemacytometry.

Phagocytosis of ^{14}C -labeled *P. aeruginosa* was measured by a modification of the method described by De Chatelet *et al.* (14). A stock culture of *P. aeruginosa* was incubated overnight at 37° in 50 ml of soybean casein digest broth containing 50 μCi of methyl ^{14}C -labeled L-amino acid mixture (New England Nuclear Corp., Boston, Mass.). The resulting ^{14}C -labeled *P. aeruginosa* was washed four times in 0.9% saline and the final bacterial pellet was dispersed in HBSS to a concentration of $10\text{--}10^8$ bacteria/ml and stored at 4° until use. *In vitro* phagocytosis was initiated by the addition of 2.0 ml of the alveolar macrophage suspension ($5\text{--}10 \times 10^6$ cells/ml) in to 0.5 ml of the ^{14}C -labeled *P. aeruginosa* suspension ($5\text{--}10 \times 10^8$ bacteria/ml) and supplemented with 12.5% serum in a total volume of 4.0 ml. Flasks were incubated at 37° in a metabolic bath shaker at 60 rpm. Incubations were terminated by the rapid addition of 5.0 ml of saline at 4° and centrifuged at 500g for 10 min at 4°. Cell pellets were washed in 0.9% saline to remove noningested bacteria and the resulting cells digested in 1.0 ml of 0.2 *N* NaOH for 4 hr at 80°. The digest was neutralized with 0.1 ml of acetic acid and duplicate aliquots were added to 10 ml of Scintiverse (Fisher Scientific Co., Rochester, N.Y.). Samples were counted in an Isocap 300 liquid scintillation system (Amersham/Searle, Arlington

Heights, Ill.) and phagocytic uptake was expressed as counts per minute of ^{14}C -labeled *P. aeruginosa* 15 min/ 10^7 alveolar macrophages.

The *in vitro* bactericidal activity of alveolar macrophages was determined by adding 2.0×10^7 alveolar macrophages obtained either prior to or 60 min following trauma to flasks containing 10% fresh rat serum and 2.0×10^7 *P. aeruginosa* in a total volume of 5.0 ml. Flasks containing serum and bacteria without macrophages served as background controls. All flasks were incubated at 37° in a metabolic shaking bath at 60 rpm and aliquots were removed and centrifuged at 500g for 10 min at 4°. Serial dilutions of the supernatants were incubated utilizing standard pour-plate colony counting techniques for 48 hr at 37° and the viable extracellular bacteria counted. Pellets of the alveolar macrophages were washed in sterile saline and resuspended in sterile distilled H_2O for 30 min in order to lyse the macrophages, and the viable intracellular bacteria were determined. Phagocytosis was expressed as the percentage of extracellular viable bacteria remaining at each incubation interval as compared to control flasks containing no alveolar macrophages. Bactericidal activity was expressed as the percentage of the phagocytized bacteria that remained viable.

The rate of oxygen uptake by macrophages was measured with a Clark-type electrode utilizing a Gilson oxymeter (Gilson Medical Electronics, Middletown, Wis.). O_2 consumption was measured at 37°, in HBSS containing 5.5 mM glucose, before and after the addition of heat-killed (30 min, 90°) *P. aeruginosa* preincubated in rat serum for 30 min at 37°. Oxygen uptake was expressed as micromoles of O_2 utilized per 60 min per 10^6 macrophages. The mean \pm the standard error of the mean was calculated for all studies. All nonpaired analyses were made utilizing the Student's *t* test, and a confidence limit of 95% was used for significance.

Results. Table 1 demonstrates the recovery yields for alveolar macrophages from control and post-traumatic rat lungs at both the acute (60-min) and 24-hr interval. Repeated lavage of rat lungs yielded $12.6 \pm$

0.97×10^6 cells/rat in nontraumatized controls; $6.4 \pm 0.4 \times 10^6$ cells/rat at 60 min post-trauma; and $7.5 \pm 0.5 \times 10^6$ cells/rat at 24 hr post-trauma. All lung cell populations demonstrated greater than 85% viability with mononuclear phagocytes comprising 90% of the isolated cell population. The yield (approximately 50%) was consistently and significantly ($P < 0.05$) less at both the 60-min and 24-hr post-trauma levels as compared to controls, but no difference was detected between the 1-hr and 24-hr trauma groups.

Phagocytosis of ^{14}C -labeled *P. aeruginosa* by macrophages lavaged from control rat lungs and from rat lungs 1.0 hr following nonlethal traumatic injury is represented in Table II. Bacterial phagocytosis by alveolar macrophages 1.0 hr following trauma in the absence of exogenous serum was significantly elevated when compared to control macrophages. With the addition of normal serum, bacterial phagocytosis was again greater by macrophages obtained at 60 min post-trauma. Furthermore, a stimulation of phagocytosis was obtained by both control and trauma macrophages with serum addition. The addition of serum from the traumatized rats obtained at 60 min postshock did not differ from control serum in its ability to enhance phagocytosis of *P. aeruginosa* by either control or trauma AM, indicating no difference in the opsonic capabilities of serum following NCD trauma, at least with respect to bacterial phagocytosis by lung macrophages. This latter comparative study evaluating the opsonic capacity of normal and 60-min post-trauma serum with respect to bacteria phagocytosis was critical since previous findings (7, 9) demonstrated a profound acute post-trauma opsonic deficiency in terms of nonbacterial phagocytosis. In order to determine if the pulmonary macrophage response was transient or would be sustained, a similar cellular comparison was performed at 24 hr postinjury. Table III illustrates bacterial phagocytosis by lung macrophages harvested 24 hr following sublethal trauma. In contrast to the stimulation of phagocytosis demonstrated 1 hr following trauma, bacterial phagocytosis by alveolar macrophages 24 hr post-traumatic injury was no longer elevated when compared to

TABLE I. RECOVERY YIELDS OF ALVEOLAR MACROPHAGES AS INFLUENCED BY TRAUMATIC INJURY.

Experimental groups	Animals per experimental group	Number of alveolar cells recovered ^a per rat ($\times 10^6$)
Pretrauma controls	60	12.6 ± 0.97^b
Post-trauma (1 hr)	60	6.4 ± 0.40
Post-trauma (24 hr)	24	7.5 ± 0.50

^a Rat lungs were repeatedly lavaged *in situ* with 0.2% EDTA in saline at 37° with a total volume of 20-30 ml.

^b Data are expressed as the mean \pm SEM. The 1- and 24-hr post-trauma yields are significantly ($P < 0.05$) less than controls.

nontraumatized control cells in the presence or absence of serum opsonins. Again, however, both control and trauma cells respond with an increased bacterial phagocytosis in the presence of serum.

The acute stimulation of phagocytosis by lung macrophages following traumatic injury warranted further investigations into the function of post-trauma cells, and Table IV demonstrates the oxygen consumption of control and 1-hr post-trauma alveolar macrophages in both the resting state and following the phagocytosis of opsonized heat-killed *P. aeruginosa*. Oxygen consumption was significantly elevated ($P < 0.05$) in the trauma alveolar macrophages when compared to control cells in the resting condition. Following the addition of opsonized heat-killed bacteria, macrophage oxygen consumption was stimulated 60% in control AM and 30% in post-trauma AM.

In order to correlate further this apparent acute hyperphagocytic state by the alveolar macrophages to injury with other parameters of host defense, bacterial killing was also studied. Figure 1 demonstrates the *in vitro* bactericidal activity of alveolar macrophages harvested either prior to or 1 hr post-sublethal trauma. Bacterial count determinations after 30 and 60 min of incubation indicated a significant acute increase in the ability of macrophages from traumatized animals to kill ingested bacteria. Control AM were able to phagocytize 1.4×10^7 *P. aeruginosa* by 30 min, of which less than

TABLE II. PHAGOCYTOSIS OF ^{14}C -Labeled *Pseudomonas aeruginosa* BY ALVEOLAR MACROPHAGES HARVESTED ACUTELY (1 hr) FOLLOWING TRAUMATIC INJURY.

Source of alveolar macrophage cell population ^a	Number of experiments	Bacterial phagocytosis ^b (cpm/15 min/10 ⁷ AM)		
		No serum	Normal serum	Trauma serum
Pretrauma controls	6	2007 \pm 104	4915 \pm 38 ^c	4886 \pm 341 ^c
Post-trauma (1 hr)	6	3021 \pm 333 ^d	5995 \pm 109 ^c	6097 \pm 293 ^c

^a Alveolar macrophages, $10\text{--}20 \times 10^6$, were incubated with ^{14}C -labeled *Pseudomonas aeruginosa* at a bacteria-to-cell ratio of 100:1 in a total volume of 4.0 ml at 37° at 60 rpm \pm 12% fresh rat serum. Each flask was supplemented with an average of 53,000 cpm (injected dose = 1D) of labeled bacteria.

^b Data are expressed as the mean \pm SEM.

^c Significantly different from no serum ($P < 0.01$).

^d Significantly ($P < 0.05$) different from pretrauma control levels of bacterial phagocytosis.

TABLE III. PHAGOCYTOSIS OF ^{14}C -LABELED *Pseudomonas aeruginosa* HARVESTED 24 HOURS FOLLOWING TRAUMATIC INJURY

Source of alveolar macrophage cell population ^a	Number of experiments	Bacterial phagocytosis ^b (cpm/15 min/10 ⁷ AM)	
		No serum	Normal serum
Pretrauma controls	6	11625 \pm 734	17984 \pm 300 ^c
Post-trauma (24 hr)	12	11376 \pm 386	16955 \pm 606 ^d

^a Alveolar macrophages, $10\text{--}20 \times 10^6$ were incubated with ^{14}C -labeled *Pseudomonas aeruginosa* (activity/flask = 300,000 cpm) at a bacteria to cell ratio of 100:1 in a total volume of 4.0 ml at 37°, 60 rpm \pm 12% fresh rat serum. Each flask was supplemented with an average of 130,000 cpm (injected dose = 1D) of labeled bacteria.

^b Data are expressed as the mean \pm SEM.

^c Significantly different from no serum controls ($P < 0.05$).

^d Significantly ($P < 0.05$) different from pretrauma control levels of bacterial phagocytosis.

30% were killed. In contrast, the post-trauma cells ingested 1.7×10^7 *P. aeruginosa* and killed greater than 50% of the ingested bacteria.

Discussion. These findings indicate that, rapidly following sublethal trauma, bacterial phagocytosis, oxygen consumption, and bactericidal activity by the pulmonary macrophage as tested *in vitro* are stimulated. Furthermore, bacterial phagocytosis is augmented in post-trauma alveolar macrophages in the absence of serum opsonins indicating a nonhumoral element, possibly a metabolic event, in the macrophage phagocytic activation. In fact, phagocytosis of *P. aeruginosa* remained elevated in alveolar

TABLE IV. EFFECT OF *Pseudomonas aeruginosa* PHAGOCYTOSIS ON O₂ CONSUMPTION BY ALVEOLAR MACROPHAGES HARVESTED PRIOR TO AND FOLLOWING (1 hr) TRAUMATIC INJURY.

O ₂ consumption ^b ($\mu\text{moles O}_2/60 \text{ min}/10^6 \text{ AM}$)			
Experimental model ^a	Number of experiments	Control macrophages (mean \pm SE)	Post-trauma macrophages (mean \pm SE)
Resting (no bacteria)	6	5.9 \pm 0.2	8.7 \pm 1.0
Stimulated (with bacteria)	4	9.5 \pm 0.4	11.3 \pm 1.1

^a Each flask contained 10×10^6 macrophages in HBSS supplemented with 5.5 mM glucose and incubated at 37°. The stimulated model was supplemented with 5×10^7 heat-killed *P. aeruginosa* previously opsonized in 10% fresh serum.

^b Data are expressed as mean \pm SEM.

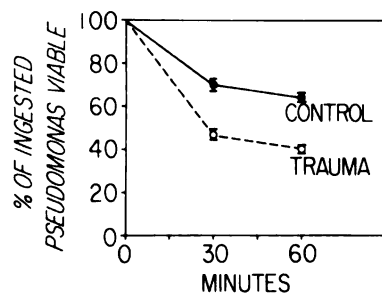


FIG. 1. The *in vitro* bactericidal activity of control (●) and 1-hr post-trauma (○) alveolar macrophages. In each flask 2.0×10^7 macrophages were incubated with 2.0×10^7 *Pseudomonas aeruginosa* in the presence of 10% rat serum at 37°. Bactericidal activity is expressed as the percentage of the calculated phagocytized bacteria that remained viable at each incubation interval. Each group consists of nine experiments with three determinations at each time interval (0, 30, 60 min).

macrophages harvested from animals 1 hr after trauma in the presence of serum opsonins, which augmented bacterial phagocytosis twofold in both populations. This stimulatory opsonic effect of serum is in close agreement with studies demonstrating a doubling of lung RE cell phagocytosis of albumin-paraffin oil emulsion particles in the presence of serum factors (15). However, the opsonic potential of serum obtained 1 hr post-trauma did not differ from control serum in its ability to enhance bacterial phagocytosis, suggesting no alterations in blood-borne bacterial opsonic factor levels in the early post-trauma period. Recent studies have indicated that hepatic RES function is maximally depressed 1–2 hr post-NCD trauma as manifested by a significant decline in the *in vivo* phagocytic index *K* value for colloid clearance. This decrease in systemic RE function has been shown to be mediated by opsonic deficiency with respect to nonbacterial phagocytosis (7, 9). The present data suggest, however, a distinct difference in pulmonary macrophage function as compared to hepatic Kupffer cell phagocytosis in the early postinjury period. Recently, Dressler and Skornik (16), utilizing a model of burn injury, reported a 27% killing efficiency by control rat AM for *P. aeruginosa* by 30 min at a bacteria to cell ratio of 50:1 and comparable to our control killing efficiency of 30% at a bacteria to cell ratio of 1:1. Furthermore, these same authors demonstrated a sustained increase in the phagocytic and bactericidal activities of rat AM following burn injury, thus lending support to the concept of pulmonary RES activation following injury. However, it appears from the present study that alveolar macrophage activation is a rapid phasic event in response to traumatic injury and appears to return to control pretrauma levels by 24 hr, at least with respect to the parameter of phagocytosis.

This temporal pattern again correlates in an inverse manner with the functional state of the Kupffer cell since it manifests phagocytic recovery after this type of trauma by 24 hr (9).

Although a wide spectrum of environmental and endogenous agents has been shown to alter the phagocytic and metabolic activities of the lung macrophage (17–19),

the metabolic activities of this cellular population during the pathogenesis of lung injury or trauma have not been explored. Increases in the postphagocytic O_2 consumption and glucose oxidation in the isolated alveolar macrophage have been reported to reflect stimulation of the HMPS pathway and H_2O_2 metabolism (4, 20). In this context, our observations demonstrate a significantly elevated O_2 consumption in the resting state in post-trauma cells. In addition, the elevated postphagocytic respiratory rate indicates a metabolic basis for the stimulation of bacterial phagocytosis in the lung macrophage following NCD trauma. The precise mechanism whereby the stimulation in O_2 consumption and H_2O_2 generation are linked to bactericidal activity in AM is presently not defined; however, Paul *et al.* (21) have recently demonstrated peroxidase- H_2O_2 activity in AM homogenates, thus delineating one potent antimicrobial system which may exist within the AM.

Rapid stimulation of macrophage phagocytosis and metabolism would be a distinct advantageous cellular defense mechanism in the lung, preventing pulmonary infection by opportunistic organisms such as *P. aeruginosa* acutely following trauma. However, one must consider the findings as presented in this study that, while on a cell-to-cell basis, there is an apparent acute activation following injury, the total number of cells recoverable was significantly reduced at both periods after trauma. Hyperphagocytosis by lung macrophages acutely following injury suggests that the previous observations of increased pulmonary localization of particulate matter following surgery in temporal association with Kupffer cell phagocytic dysfunction may not represent a non-specific event as previously suggested (9–12). This may reflect a compensatory response by extrahepatic macrophages to compensate for the hepatic RE depression or it may be mediated at the local level by the presence of tissue debris and denatured protein generated as a result of the trauma (7). In either event, the relationship of these findings to the previously documented pulmonary localization of blood-borne cellular and noncellular particulate matter following trauma (7) warrants investigation.

Summary. The *in vitro* phagocytic and

idal responses of rat alveolar macrophages were investigated following sublethal traumatic shock. Phagocytosis of ^{14}C -*Pseudomonas aeruginosa* by lung macrophages was elevated 1 hr post-trauma in the absence of bacterial opsonins. This response was transient with a return to normal by 24 hr. Bacterial phagocytosis by control trauma alveolar macrophages was reduced in the presence of serum obtained prior to or following trauma. In association with this acute phagocytic activation, O_2 consumption as well as bactericidal activity was stimulated in isolated alveolar macrophages harvested at 60 min post-traumatic injury. In contrast, the relative yields of lung macrophages following traumatic injury were significantly reduced at both the 1-hr and 24-hr postinjury period. Alveolar macrophage phagocytic and metabolic metabolism are thus rapidly and transiently activated following host defense mechanisms following sublethal trauma. This response is in direct contrast to the previously documented acute depression of alveolar Kupffer cell following traumatic injury and may represent a compensatory mechanism of the lung RES during periods of macrophage RES dysfunction.

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Thyroid and Blood Thyrocalcitonin Concentrations and C-Cell Abundance in Strains of Rats at Different Ages¹ (39525)

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Previous reports of changes in thyrocalcitonin (TCT) status in rats of different ages are conflicting. Some workers have reported that thyroid TCT levels increase with age (1) whereas others have observed (a) no differences in thyroid TCT in 60-, 120-, and 360-day-old rats (2) and (b) no difference in blood TCT between young and old rats (3). Since our own earlier studies employing the bioassay for TCT showed differences in the thyroid gland content of TCT between age-matched rats of different strains (4), and since the earlier conflicting studies (1-3) employed rats of different strains as well as different ages, we decided to examine the question of possible changes in TCT in young and old rats from two different strains commonly used in our laboratory.

Using a recently developed immunoperoxidase technique for light microscopic localization of rat thyroid C-cells (5) and an improved radioimmunoassay for rat TCT (6), we have evaluated and compared the relative area ratio between C-cells and follicular cells and the thyroid and blood TCT concentration in Fischer inbred rats (F344) and Holtzman rats.

Material and methods. Animals. Holtzman albino rats (descended from a Sprague-Dawley strain) were purchased from the Holtzman Co., Madison, Wis.; Fischer inbred rats (F344) were obtained from the Charles River Breeding Labs., Wilmington, Mass., (Charles River CDF rats). Following receipt, the rats were maintained on Purina

laboratory chow and tap water.

Histology. Preparation of tissues and immunohistochemical staining have been described previously (5). The immunoperoxidase technique revealed brown-stained cells which were easily distinguished from unstained follicular cells. Comparison of area ratios of these two types of thyroid cells was performed according to the procedure described by Chalkley (7). An occluding micrometer consisting of 5 horizontal and 20 vertical lines was attached to an eyepiece (10×), and the 100 points where the horizontal lines intersected the vertical lines were used as finders. The intersections constituted the point pattern. The number of C-cells and follicular cells in tissue sections that fell on these points were recorded as "number of hits." Cross sections obtained at the midline of the thyroid, in addition to the caudal and distal poles of the thyroid gland, were used, because the midline area has the highest concentration of C-cells (8). The total area of the cross section was measured with the micrometer, and the results were expressed as the area ratio between C-cells and follicular cells. Tissues for electron microscopic studies were prepared and processed as described previously (9).

Blood collection and analysis. Genital blood was obtained from each rat by puncture and allowed to clot. Serum was separated by centrifugation within 2 hr of blood collection. The serum levels of TCT and the thyroid gland content of TCT were measured by radioimmunoassay as described previously (6).

Statistical analysis. Experimental data were subjected to analysis of variance. Standard errors were calculated from the usual error term of the analysis of variance. The significance of differences between

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values was evaluated either by the F or by a two-tailed t test. For the data in Fig. 1, standard linear regression was employed with body weight as the independent variable (X) and thyroid weight the dependent variable (Y). The value for the correlation coefficient (r) was calculated and analyzed for chance (P) by a 2-tailed test.

Results. Table I shows the TCT concentrations in thyroid glands and peripheral blood of 10-week-old Fischer rats and Holtzman rats. The mean thyroid TCT concentration in Fischer rats was slightly higher than in Holtzman rats, but the difference was not statistically significant. The level in the peripheral blood was below ng/ml except for two out of eight Fischer rats and one of eight Holtzman rats. Table II shows results of three experiments (A, B, and C) in which TCT concentrations in the thyroid glands and in the peripheral blood of 6- to 18-month-old rats of the two strains were measured. Results obtained from 6- and 18-month-old rats were combined for presentation since no differences between these two ages were found.

In Experiment A, serum TCT was higher in Fischer rats than in Holtzman rats,

TABLE I. TCT CONCENTRATIONS IN THYROID GLANDS AND PERIPHERAL BLOOD OF 10-WEEK-OLD FISCHER AND HOLTZMAN RATS.

	Fischer	Holtzman
Thyroid (ng/mg)	86 \pm 13.4 ^a	54 \pm 13.4
Serum (ng/ml)	6 of 8 = N.D. ^b (2 of 8 = 0.31, 0.49)	7 of 8 = N.D. (1 of 8 = 0.29)

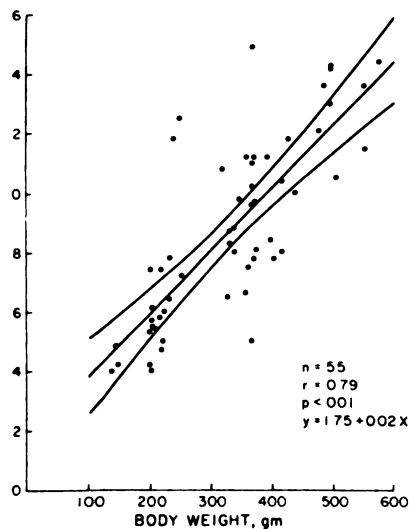
^a Values of TCT concentration in thyroid glands are shown as mean \pm SE ($N = 8$).

^b N.D. = not detectable or <0.24 ng/ml.

and this difference was highly significant ($P < 0.001$). In Experiment B, TCT concentrations in both peripheral blood and thyroid glands were measured in Fischer rats. A high serum TCT in Fischer rats, similar to that found in Experiment A, was associated with a high thyroid TCT concentration. In Experiment C, TCT concentrations in peripheral blood and thyroid glands were measured in Holtzman rats. A low serum TCT level, similar to that obtained in Experiment A, and a low thyroid TCT concentration were found.

Figure 1 shows the positive correlation observed between the body weight of the rats used and the wet weight of one of their two thyroid lobes ($1/2$ -thyroid) which was analyzed for TCT. Figure 2 shows results of thyroid weight and TCT content measurements in 10-week-old rats. The $1/2$ -thyroid weight of Holtzman rats and Fischer rats was different, but no significant difference in $1/2$ -thyroid TCT was found between Holtzman and Fischer rats. Figure 3 shows the thyroid weight and TCT content in 6- to 18-month-old rats of the two strains. Again the $1/2$ -thyroid weights of Holtzman rats and Fischer rats were different ($P < 0.001$). The $1/2$ -thyroid TCT content also was different ($P < 0.05$).

We also examined the distribution of C-cells in Fischer rats. In 10-week-old rats, C-cells were localized chiefly in the central position of the thyroid lobes, a distribution similar to that which we described previously for 4- to 5-week-old Holtzman rats (5). In 6- to 18-month-old Fischer rats, there was a significantly higher area ratio of C-cells to follicular cells (0.15 ± 0.026 ;



1. Positive correlation between body weight and thyroid weight of both Fischer and Holtzman rats. Between the two convex curves represents 95% confidence limits of the regression line.

TABLE II. TCT CONCENTRATIONS IN THYROID GLANDS AND PERIPHERAL BLOOD OF 6- TO 18-MONTH-OLD FISCHER AND HOLTZMAN RATS.

Experiment	Fischer		Holtzman	
	Serum (ng/ml) ^a	Thyroid (ng/mg) ^a	Serum (ng/ml) ^a	Thyroid (ng/mg) ^a
A	1.52 ± 0.06	—	0.68 ± 0.06	—
B	1.37 ± 0.06	461 ± 38	—	—
C	—	—	0.48 ± 0.06	182 ± 38

^a Values of TCT concentration in serum and thyroid glands are shown as mean ± SE.

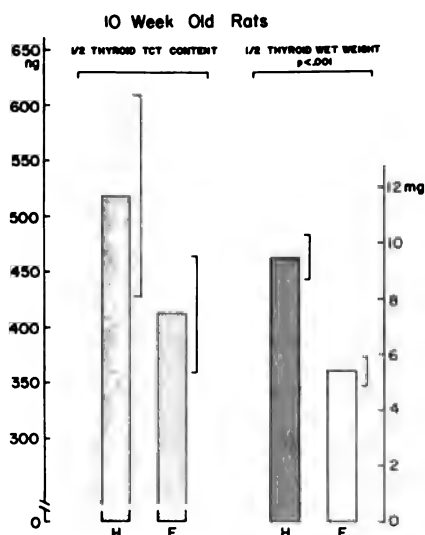


Fig. 2. TCT in thyroid glands of 10-week-old Holtzman rats (H) and Fischer rats (F). The two bars on the right represent the mean weights of one thyroid lobe (1/2-thyroid gland) and the two bars on the left show the total TCT content obtained in those thyroid lobes (1/2-thyroid TCT content). Brackets denote ± SE.

mean ± SE) than in 10-week-old Fischer rats (0.08 ± 0.013). Electron microscopic study of thyroid tissue from an 18-month-old Fischer rat showed abundant clusters of C-cells which were easily found (Fig. 4). Like C-cells in young rats, they were located intrafollicularly close to the basal membrane.

Discussion. The results show that in Fischer and Holtzman rats, most of the 10-week-old animals had undetectable levels of blood TCT (0.24 ng/ml , Table I). In contrast, blood TCT of all older rats from both strains was much higher and easily measured (0.5 – 1.5 ng/ml , Table II). Our findings are not in agreement with a recent report showing no differences in blood TCT

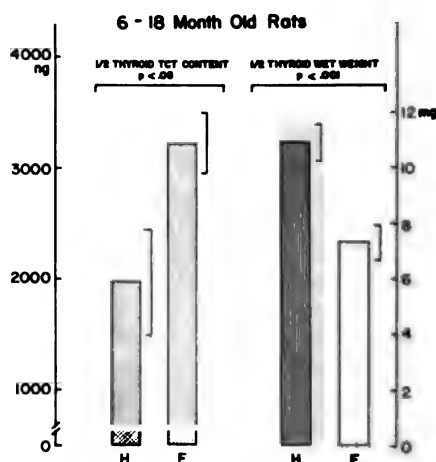


Fig. 3. TCT in thyroid glands of 6- to 18-month-old Holtzman rats (H) and Fischer rats (F) are shown. For details see legend to Fig. 2.

between young (120–140 g) and old (2 years) Wistar rats (3). These discrepancies may be due to strain differences, since in our study a significantly ($P < 0.001$) higher blood TCT was observed in Fischer rats compared with Holtzman rats of the same age (Table II, Experiment A). Not only the concentration of TCT in blood but also the concentration of TCT in thyroid tissue increased with age (Tables I and II). Since the size (weight) of the thyroid glands also increased with age (body weight) (Fig. 1), we also examined the relationship between thyroid weight and thyroid TCT content (Figs. 2 and 3). In 6- to 18-month-old rats, the results clearly showed that Fischer rats had smaller thyroid glands but a higher TCT content than Holtzman rats (Fig. 3). It has been reported previously that in Wistar rats thyroid TCT content increases with age (1). However, it is not clear whether these earlier results are in agreement with our present findings or whether the apparent in-

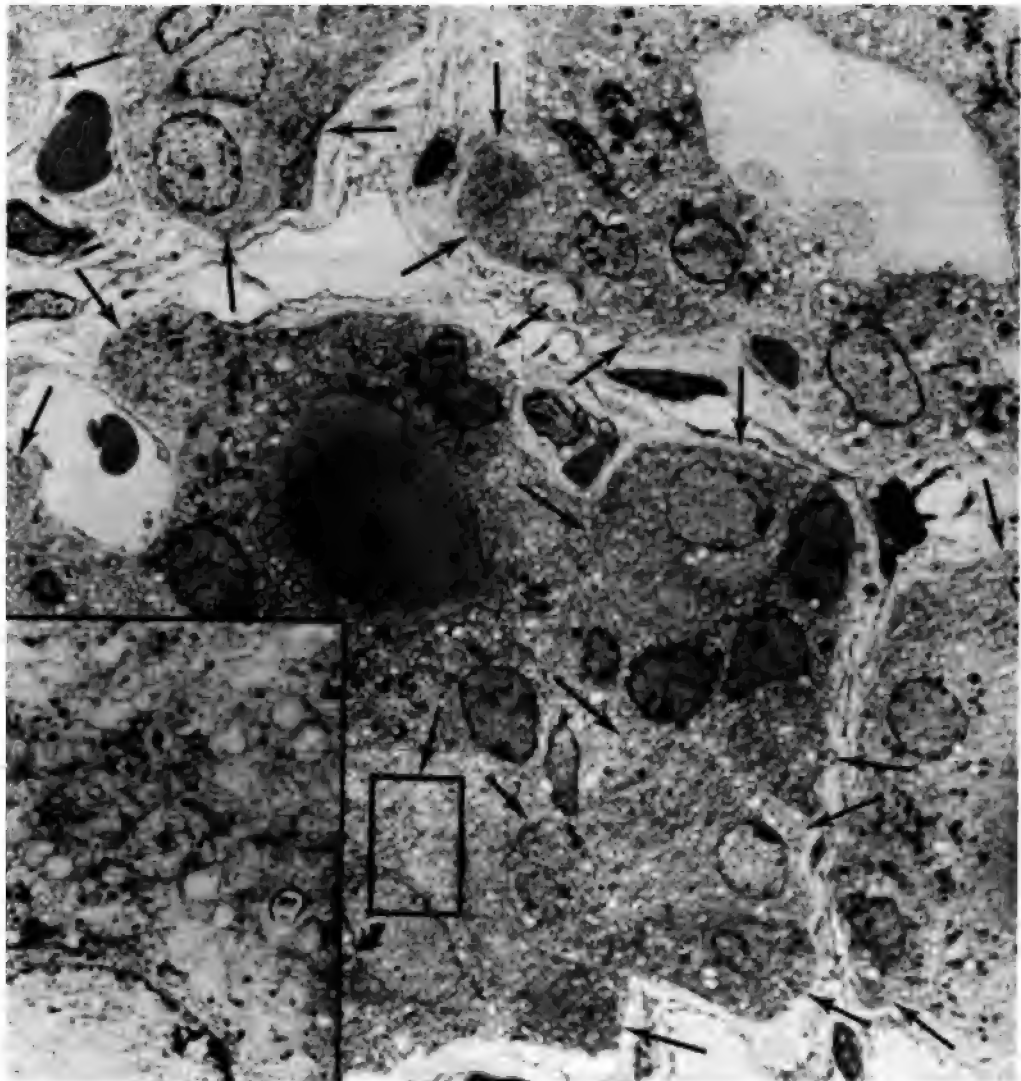


FIG. 4. Electron micrograph (montage) of thyroid tissue from an 18-month-old Fischer rat shows abundant C-cells readily identified by the presence of secretory granules. C-cells (arrows) are found intrafollicularly and are concentrated close to the basal membrane (uranyl acetate and lead citrate; montage $\times 2430$; inset $\times 9000$).

increase in thyroid TCT content reported was due merely to an increase in the size of the thyroid glands with increasing age of the rats. In contrast, other investigators have not found differences in thyroid TCT content, as measured by bioassay in 60-, 120-, or 360-day-old Sprague-Dawley rats (2). We have used male and female rats of both strains in our present study. The data were combined since no sex differences with respect to blood and thyroid TCT were observed.

Because of the high level of TCT in blood and in the thyroid gland, Fischer rats may represent an especially useful strain of rat for the study of TCT secretion. The significance of the differences in TCT between Fischer rats and Holtzman rats and the factors responsible for the increases in TCT in blood and thyroid gland with age remain to be elucidated.

Summary. We have shown an increase in TCT in the blood and thyroid gland with increased age in two different strains of rats.

Fischer (F344) rats, 6 to 18-months old, have a higher level of circulating TCT (~1.5 ng/ml) than young 10-week-old Fischer rats (<0.2 ng/ml). Thyroid TCT concentrations in old Fischer rats also were significantly higher than in young Fischer rats, and this was associated with a significantly higher area ratio of C-cells to follicular cells in the older rats. In Holtzman rats a similar increase in blood and thyroid TCT with age was also observed. Comparing the two strains, both thyroid and serum TCT in old Fischer rats were significantly higher than in old Holtzman rats.

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vated Methionine-tRNA Synthetase Activity in Human Colon Cancer¹ (39526)

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e incorporation of all amino acids into
ins requires two steps: first the forma-
of aminoacyladenylates and then the
hment of the aminoacyl moiety to a
fic transfer RNA (tRNA). For each
o acid both of these reactions are cata-
by an aminoacyl-tRNA synthetase
is specific for that amino acid (1). The
ions may be written as follows.

mino acid + ATP + enzyme \rightleftharpoons
aminoacyladenylate·enzyme + PP_i.

aminoacyladenylate·enzyme + tRNA
aminoacyl-tRNA + AMP + enzyme.

However, in both prokaryotic and
ryocytic cells the initiation of protein
esis requires the participation of a spe-
minoacyl-tRNA_f. The tRNA involved
tiation (tRNA_f^{met}) differs structurally
the tRNA involved in the insertion of
ionine into growing polypeptides
A_f^{met}) (2, 3) but the synthesis of both
tRNAs is catalyzed by the same en-
Met-tRNA synthetase (4).

any studies have demonstrated high
of protein synthesis in rapidly growing
rs (5) and, in some animal models,
in synthesis increases in normal tissue
to the development of neoplastic
formation (6). Increased activity of
oacyl-tRNA synthetases would be ex-
ed in conjunction with increased protein
esis, and DelMonti and Cini have dem-
ated increased activity of a number of
oacyl-tRNA synthetases, including
tRNA synthetase, in rat hepatomas as

compared to normal rat liver (7). Enzyme
activities correlated with the growth rates of
the tumor.

In the studies presented herein, Met-
tRNA synthetase activity was measured in a
human neoplasm. Colon cancer was chosen
as the test tumor since a large amount of
normal colon is generally excised at the time
of surgical resection of the tumor. Thus it is
possible to compare enzyme activity in the
tumor with the adjacent normal tissue. In
addition, experiments were done to study
the inhibitory effects of a synthetic aminoal-
kyladenylate, methioninyladenylate, on
mammalian Met-tRNA synthetase.

Materials and methods. Human colons
containing well-differentiated adenocarci-
nomas were obtained at the time of surgical
resection. Normal colonic mucosa was sepa-
rated from the underlying tissue by sharp
dissection, rinsed in chilled saline, and im-
mediately homogenized in 0.25 M sucrose
which contained 0.01 M Tris, pH 8.0, 0.5 M
KCl, and 0.01 M MgCl₂. The homogenate
was centrifuged 90 min at 67,000g. The
most superficial, intraluminal portion of the
carcinomas was separated from the underly-
ing fibrous stroma of the tumors by sharp
dissection and then similarly processed. The
particle-free supernates were collected with
a syringe and the protein content deter-
mined by a modification of the procedure of
Lowry *et al.* (8) using beef serum albumin as
a standard. Aliquots of the supernatants (1-
20 μl) were then incubated with a mixture
of yeast tRNAs (1 mM; Calbiochem, La
Jolla, Calif.), [³H]methionine (2.7 μM,
10,000 cpm; New England Nuclear, Bos-
ton, Mass.), ATP (5 mM); Sigma Chemical
Co., St. Louis, Mo.), dithioerythritol (5
mM), KCl (100 mM), MgCl₂ (10 mM), and
Tris buffer (50 mM), pH 7.0.

Solutions were incubated for 15 min at
37°, then quickly chilled and 75 μl were
transferred to a paper circle 23 mm in diam-

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Salt Lake City, Utah 84132.

eter (Whatman 3MM). This was immediately plunged into ice-cold 5% trichloroacetic acid (TCA). After 15 min, the pad was washed thoroughly with cold 5% TCA and then with ethanol:ether (1:1). The pad was then immersed in cold ether for 15 min, dried, placed in a vial with 10 ml of a scintillation cocktail [Omnifluor (New England Nuclear) in toluene, 4 g/liter], and counted in a liquid scintillation counter. The specific activity of Met-tRNA synthetase was then calculated and expressed as nanomoles of Met-tRNA formed per minute per milligram of protein. Under these conditions the amount of product formed is proportional to enzyme concentration and to time.

In experiments done to study the inhibitory effects of methioninyladenylate, the source of Met-tRNA synthetase was a particle-free homogenate of rabbit liver or human colon tumor which was prepared as above. Methioninyladenylate was prepared by a modification of the method of Cassio *et al.* (9). Incubation mixtures were as above but in addition contained methioninyladenylate in concentrations ranging from 2.16×10^{-8} to 2.16×10^{-5} M. Experiments were done utilizing two concentrations of [3 H]methionine, 2.7 and 5.4 M. All assays were performed in duplicate.

Results. Met-tRNA synthetase activity in normal and carcinomatous colonic mucosa. The Met-tRNA synthetase activity of normal colonic mucosa and colonic cancers from five patients is shown in Table I. The protein concentration of the particle-free extracts from the normal and cancerous tissues was similar (normal mean = 12.4 mg/ml; carcinoma mean = 11.23 mg/ml). In each case specific enzymatic activity in the cancer exceeded that in the adjacent normal mucosa by a factor of at least 2:1. The mean

specific activity of Met-tRNA synthetase in the tumor tissue was approximately four times that of the normal mucosa. This is a highly significant difference ($P < 0.05$, two-tailed).

Inhibition of mammalian Met-tRNA synthetase by methioninyladenylate. Methioninyladenylate proved to be a potent inhibitor of rabbit hepatic Met-tRNA synthetase. In assays utilizing 2.7 M methionine as the substrate there was detectable inhibition of Met-tRNA synthetase activity at concentrations of methioninyladenylate of 10^{-8} M (Table II). Concentrations of methioninyladenylate of 10^{-5} M almost completely inhibited enzymatic activity (Table II). As expected with a competitive inhibitor, a higher substrate concentration (5.4 M methionine) partially blocked the inhibitory effect of methioninyladenylate at 2.2×10^{-8} M, the lowest concentration studied (Table II). Similar results were obtained with extracts of normal colonic mucosa and carcinomatous mucosa (83% inhibition at 2.2×10^{-6} M methioninyladenylate).

Discussion. The reaction catalyzed by the enzyme Met-tRNA synthetase can be considered as the first committed step of protein

TABLE II. INHIBITION OF MET-tRNA SYNTHETASE BY METHIONINYLADENYLATE.^a

Methioninyladenylate (M)	[3 H]Met-tRNA formed ^b from 2.7 M Met	Inhibition (%)	[3 H]Met-tRNA formed ^b from 5.4 M Met	Inhibition (%)
0	112.0		162.0	
2.2×10^{-8}	98.6	12	161.0	1
2.2×10^{-7}	76.1	23	127.0	22
2.2×10^{-6}	22.3	80	41.3	75
2.2×10^{-5}	3.6	97	6.8	96

^a Met, methionine.

^b Nanomoles per assay tube.

TABLE I. SPECIFIC ACTIVITY OF MET-tRNA SYNTHETASE IN EXTRACTS FROM NORMAL COLONIC MUCOSA AND COLONIC CANCERS^a

	Case number					Mean
	1	2	3	4	5	
Colonic cancer	0.32	0.25	1.26	0.48	0.60	0.58
Normal mucosa	0.11	0.12	0.29	0.15	0.08	0.15
Cancer/normal	2.9	2.1	4.3	3.2	7.5	3.9

^a Specific activity reported as nanomoles of Met-tRNA per minute per milligram of protein. Met, methionine.

esis. Methionine is not available for the metabolic pathway once it is attached to the initiator tRNA. Rapidly growing cells require increased rates of synthesis, and thus the findings herein, i.e., increased activity of Met-tRNA synthetase in human colon cancer compared with normal colonic mucosa support the concept that the amount of free aminoacyl-tRNA plays a significant rate-controlling role in cell growth (10). It is very likely that Met-tRNA synthetase activity is increased in other human neoplasias as well. This tissue is known to take up methionine avidly and this fact is utilized in the treatment of ^{75}Se -labeled methionine analogs in the detection of a variety of tumors (11, 12).

Previous studies have shown that methioninyladenylate inhibits the activity of Met-tRNA synthetase in both bacterial and avian systems *in vitro* (13, 14). This inhibitory effect is on a nonlethal condition of suspended growth which can be reversed by the addition of methionine to the culture media (13, 14). Our results indicate that mammalian Met-tRNA synthetase is also inhibited by methioninyladenylate. This finding suggests the possibility of synchronizing populations of tumor cells by the sequential administration of methioninyladenylate and methionine. Maintaining inhibitory concentrations of methioninyladenylate *in vivo* may be difficult, were it possible, the synchronization could enhance the cytotoxic capacity of cycle active antitumor drugs.

Materials and Methods. Methionine-tRNA synthetase was purified from the formation of methionine-Met-tRNA (Met-tRNA) which is necessary for initiation of protein biosynthesis. This compares Met-tRNA synthetase activities of human colon cancer and normal colonic mucosa.

Cell-free supernatants were prepared from homogenates of tumors and adjacent normal mucosa. These preparations were incubated with ATP, a mixture of yeast Mg^{2+} , K^+ , dithioerythritol, and methionine. Specific activities (nanomoles of $[^3\text{H}]\text{Met-tRNA}$ per milligram of Met-tRNA synthetase from tu-

mors and normal mucosa were measured and compared.

Five patients were studied and in each the tumor sample had a higher specific activity than its normal counterpart. The mean specific activity of Met-tRNA synthetase in the tumor tissue was approximately four times that of the normal mucosa. Methioninyladenylate, when added to the assay tubes, proved to be a potent inhibitor of Met-tRNA synthetase.

Since protein biosynthesis begins with the formation of Met-tRNA, the synthetase may be a rate-limiting enzyme governing protein biosynthesis in tissues. High specific activity of the enzyme would be expected to correlate with rapid cell growth. Inhibition of the enzyme with methioninyladenylate may prove a useful method of regulating protein biosynthesis in tumor cells.

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ce that Secretin Does not Have Direct Antitrophic Effects on the Rat Stomach (39527)

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discovery that gastrin stimulates the of the pancreas and gastrointestinal opened up a whole new area of ation in gastrointestinal physiology. at time the other two "classical" testinal hormones, cholecystokinin etin, have been tested to determine they, too, have trophic effects on es of the digestive tract (1). There is isiderable evidence to suggest that tokinin does, indeed, have trophic at least on the exocrine pancreas. In of secretin the story is not as clear. ng that is clear, however, is that can block the trophic effect of pen- on the rat stomach. This was first trated by Stanley *et al.* in 1972 when wed that pentagastrin-induced pari- hyperplasia in the rat can be pre- if secretin is given simultaneously pentagastrin (2). In a more recent ohnson and Guthrie reported that strin-stimulated DNA synthesis *in* the rat stomach and small intestine e prevented if secretin is given eously with the pentagastrin (3). ibition of pentagastrin-stimulated y secretin suggests that, instead of . trophic effect, secretin may, if any- ave an antitrophic effect on the gas- inal tract of the rat. The purpose of ent study was to determine whether , when given alone, inhibits the of the rat stomach. The answer to stion is not only important in terms standing the physiology of gastroin- hormones but takes on added signif- view of the considerable potential tin as an anti-ulcer drug.

ials and methods. Twenty-four male -Dawley rats, weighing between 225 g, were divided into two equal One group was injected subcutane- very 8 hr with synthetic secretin z/Mann Inc., Orangeburg, N.Y.) at

a dose of 100 units/kg. This dose of secretin is identical to that used by Stanley *et al.* (2) to block pentagastrin-induced parietal cell hyperplasia and slightly larger than the dose used by Johnson and Guthrie (3) to block pentagastrin-stimulated DNA synthesis. The injected secretin was dissolved at a concentration of 10 $\mu\text{g/ml}$ in a gelatin solution consisting of nine parts of calcitonin diluent B (Armour Pharmaceutical Co., Kankakee, Ill.) and one part of 0.01 *N* HCl. The second group of rats was injected with only the gelatin solution and served as controls. All rats were allowed free access to water and a stock diet of Lab-Blox (Allied Mills Inc., Chicago, Ill.).

After 14 days of treatment the rats were etherized and killed by exsanguination. Secretin had no effect on the final body weight of the rats used in this study. Blood samples were drawn from the heart of each rat and centrifuged at 3000*g* for 15 min. The resultant serum samples were stored at -20° until assayed for gastrin content. The oxyntic glandular mucosa was isolated from each stomach by scraping it free from the underlying serosa using a glass microscope slide. Each mucosal scraping was homogenized in 3 ml of ice-cold water, and the resultant homogenates were centrifuged at 3000*g* for 15 min. The supernatant solutions were assayed for carbonic anhydrase activity as well as for pepsinogen and protein content. Nucleic acids and protein were extracted from the remaining pellet as previously described (4).

Samples assayed for pepsinogen content were adjusted to pH 8.0 with 0.01 *N* NaOH and allowed to stand on ice for 10 min. During this time, the pepsin initially present in the samples was permanently inactivated by the high pH. The samples were then adjusted to pH 2.0 with 0.01 *N* HCl, converting the pepsinogen present to pepsin. This newly activated pepsin was assayed by

the method of Anson (5). Carbonic anhydrase was assayed by the electrometric method of Wilbur and Anderson (6). DNA and protein were determined by the methods of Burton (7) and Lowry *et al.* (8), respectively.

Antral gastrin was extracted by homogenizing each antrum in 2 ml of ice-cold distilled water. One milliliter of homogenate was added to 4 ml of boiling water, and the mixture incubated at 100° for 20 min. The mixtures were then centrifuged at 3000g for 15 min, and the resultant supernatant solutions were filtered by vacuum through 0.45- μ m Millipore filters. The filtrates were stored at -20° until assayed for gastrin content. Gastrin was assayed in the serum samples and the antral filtrates by radioimmunoassay as previously described (9).

Results and discussion. We examined the effect of multiple secretin injections on the weight of the stomach and its component tissues, on the biochemical characteristics of the oxyntic glandular mucosa, and on the levels of endogenous gastrin. Secretin had no effect on the weight of the stomach, oxyntic gland area of the stomach, antrum, or oxyntic glandular mucosa (Table I). The DNA, protein, and pepsinogen contents as well as the carbonic anhydrase activity of the oxyntic glandular mucosa were also unaffected by secretin (Table II). In addition, secretin had no effect on antral or serum gastrin levels (Fig. 1).

Each of the measurements made in this study is capable of detecting changes in the growth of the stomach. Any changes occurring in gastric growth would, of course, be reflected by a similar change in the weight of the stomach or one of its component tissues. Both DNA and protein content have been used previously to demonstrate the trophic effect of gastrin on the rat gastrointestinal mucosa and are thus proven indices of mucosal growth (10, 4). In the stomach, pepsinogen is found primarily in chief cells. Consequently, the pepsinogen content of the oxyntic glandular mucosa should be an indication of the number of chief cells present. Similarly, Davenport has found that an excellent correlation exists between the carbonic anhydrase content and the parietal cell mass of the stomach (11). Therefore, both pepsinogen content and carbonic anhy-

TABLE I. WEIGHT OF THE RAT STOMACH AND COMPONENT TISSUES AFTER MULTIPLE SECRETIN INJECTIONS.^a

Tissue	Secretin	Control
Stomach (mg/100 g body wt)	556 \pm 12 ^b	559 \pm
Oxyntic gland area (mg/100 g body wt)	341 \pm 9	338 \pm
Antrum (mg/100 g body wt)	59.9 \pm 2	57.6 \pm
Oxyntic glandular mucosa (mg)	440 \pm 37	386 \pm

^a 100 units/kg injected subcutaneously every 8 hr for 14 days.

^b Standard error of the mean.

TABLE II. BIOCHEMICAL CHARACTERISTICS OF T OXYNTIC GLANDULAR MUCOSA AFTER MULTIPLE SECRETIN INJECTIONS.^a

Measurement	Secretin	Control
DNA (μ g/g mucosa)	37.4 \pm 3.0 ^b	39.2 \pm 4.
Protein (mg/g mucosa)	7.88 \pm 0.4	7.43 \pm 0.
Pepsinogen (pepsin/mg mucosa) ^c	2.04 \pm 0.19	2.02 \pm 0.
Carbonic anhydrase activity (units/mg protein)	19.6 \pm 1.3	19.6 \pm 1.

^a 100 units/kg injected subcutaneously every 8 hr for 14 days.

^b Standard error of the mean.

^c Pepsinogen is expressed as equivalent micrograms of activated pepsin.

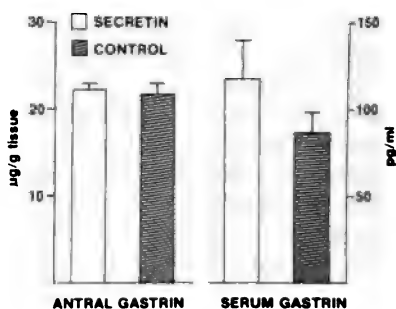


FIG. 1. Endogenous gastrin levels of rats after multiple secretin injections. Secretin was injected subcutaneously every 8 hr for 14 days at a dose of 100 units. Each bar represents the mean \pm SE of 12 rats.

drase activity should provide information about changes occurring in the cell populations of the gastric mucosa. Endogenous gastrin levels are also an index of mucosal growth. For example, the low antral

gastrin levels observed in antrectomized rats (10), starved rats (9, 12), and rats maintained by total parenteral nutrition (4) accompanied in each case by a reduction in growth of the gastrointestinal mucosa. Furthermore, the high levels of endogenous gastrin observed in patients with Zollinger-Ellison syndrome are associated with an increase in mucosal growth (13, 14).

Although each measurement in this study was capable of detecting changes in stomach weight, not one measurement provided any evidence of an inhibitory role for secretin. Secretin did not decrease the carbonic anhydrase activity or, presumably, the parietal cell mass of the oxyntic glandular mucosa although Stanley *et al.* have previously reported that secretin induces parietal cell hyperplasia in the rat stomach (2). There might not be a conflict in results, however, since the decrease in parietal cell mass reported by these workers was not significant in every experiment. Our findings agree with those of Johnson and Guthrie, who were unable to demonstrate an inhibitory effect of secretin on *in vitro* DNA synthesis in the rat stomach (3). We conclude that secretin itself does not have an antitrophic effect on the rat stomach and that the previously reported antitrophic effect of secretin on this tissue (2, 3) was due to the inhibition of pentagastrin.

The findings of this study do not, of course, rule out an antitrophic role for secretin in the other tissues of the digestive tract. Pansu *et al.* recently reported that secretin can prevent a nocturnal increase in cell proliferation in the rat jejunum (15). The nocturnal increase in cell proliferation is part of a circadian rhythm in mitosis that exists in this tissue. Thus, secretin may well have an antitrophic effect on the rat small intestine.

Method. Rats were injected subcutaneously with synthetic secretin (100 units/kg) 8 hr for 14 days. We examined the effect of secretin on the weight of the stomach and its component parts, on the biochemical characteristics of the oxyntic glandular mucosa, and on the levels of endoge-

nous gastrin. Each of these measurements is capable of detecting changes in the growth of the stomach. Nevertheless, we found no evidence that secretin inhibits gastric growth. It is, therefore, concluded that secretin itself does not have an antitrophic effect on the rat stomach and that the previously reported antitrophic effect of secretin on this tissue was due to the inhibition of pentagastrin.

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A New Method for the Establishment of Diploid Fibroblast Cell Cultures from Human Foreskins¹ (39528)

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Human diploid fibroblast cultures are a useful source of cells for many areas of investigation in biochemistry, virology, and genetics. These cultures have usually been established from skin biopsies according to the method of Martin (1) which involves mincing the piece of skin and allowing the fragments to adhere to the bottom of small culture vessels, after which the cells explant. An easily available source of human skin is the foreskin obtained from routine circumcisions, and cultures from these can be established from explants of the minced tissue. Since this source yields a relatively large amount of tissue, it should be convenient for digestion procedures. Methods involving trypsinization of tissue before establishing fibroblast cultures have been reported using embryonic lung and other organ sources (2, 3). A complex digestion procedure for the culture of foreskin, reported by Pious *et al.* (4), yielded a small amount of cells for clonal growth studies. The present communication presents a method for deriving a larger yield of fibroblast cultures from foreskins by a simple trypsin digestion. The new procedure circumvents the explanation of large amounts of minced tissue fragments and yields large numbers of cultured cells in a short period of time.

Methods. Foreskins were obtained with informed consent, and with approval of the Institutional Human Studies Committee, from routine circumcisions performed at the

Philadelphia General Hospital and at the Medical College of Pennsylvania. Foreskins were collected in Dulbecco's phosphate-buffered saline supplemented with glucose (1 mg/ml), gentamicin (50 μ g/ml), amphotericin B (2.5 μ g/ml) and Mycostatin (100 U/ml). Foreskins can remain chilled in this saline for at least 24 hr before processing for culture. After rinsing and removing any adherent adipose tissue, foreskins (average wet weight, 375 mg) were minced and torn into small pieces using sharp scissors and scalpel. This was accomplished in a minimal volume of saline. The minced foreskin was then transferred to 40 ml of a 0.25% trypsin solution in minimal essential medium (MEM) supplemented with amphotericin B (2.5 μ g/ml), gentamicin (50 μ g/ml), and Mycostatin (100 U/ml), and the pH was adjusted to 7.4. The trypsin mixture was agitated slowly at room temperature for 24 hr, or at 37° for 6 hr. The digest mixture was then filtered through lens paper to remove large pieces of undigested tissue. The filtrate was centrifuged at 500g for 10 min, and the resultant cell pellet was dispersed in Ham's medium F-12 supplemented with 20% fetal bovine serum and penicillin (50 U/ml) and streptomycin (50 μ g/ml). Cells were plated in 50-cm² culture vessels containing 10 ml of medium. The medium was replaced within 3 days and at weekly intervals thereafter. When cultures were confluent they were subcultivated using 0.05% trypsin solution in Ham's saline (5); the cells were then grown in a less complete medium such as MEM supplemented with 10% serum.

Results and discussion. By 24 hr, fibroblastic cells could be seen adhered to the vessel surface; in addition, there was a large number of spherical cells, which both adhered to the surface and floated in the me-

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dium. These latter cells could be injured cells or epithelial cells isolated by the trypsin digestion; they did not grow. The typical appearance of the initial culture after the medium change on Day 3 is indicated in Fig. 1a. The fibroblasts had begun to proliferate, and some rounded cells remained attached to the surface. The fibroblastic cells had morphological growth patterns typical of fibroblast cultures (2, 6), and they became confluent within 10 days to 3 weeks. A

typical confluent monolayer at that time is shown in Fig. 1b. The method can yield approximately 10^7 cells per foreskin within 2 weeks.

The storage of the foreskins in the antibiotic-containing saline allowed a delay of at least 24 hr before processing. This procedure also probably aided in decontaminating the foreskin. The majority of the fibroblasts is probably derived from the dermal layer which immediately underlies the epi-

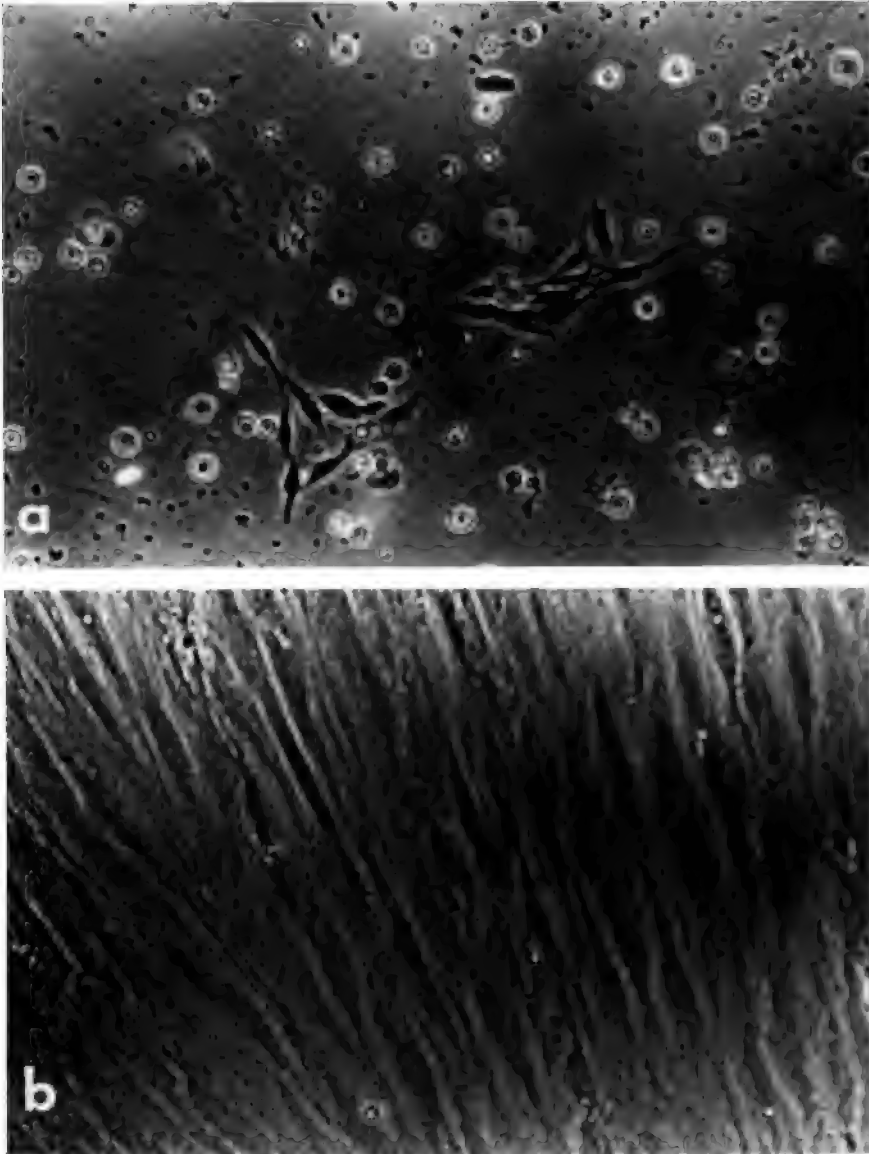


FIG. 1. (a) Phase photograph of human foreskin culture on third day after digestion ($\times 224$). (b) Phase photograph of confluent monolayer of human foreskin fibroblasts ($\times 224$).

dermis. However, it did not seem necessary, and in fact it was technically very difficult, to separate the epidermal layer by dissection before incubation. The careful mincing in very small pieces appeared to ensure adequate digestion of the dermal layer.

A number of different enzymes and media was tried. Neither collagenase nor Pronase resulted in as efficient a cell dissociation as did the standard cell culture with trypsin. The inclusion of antibiotic and antimycotic agents at the stated levels did not seem to inhibit cell yield or growth. Digestion in complete medium at constant pH resulted in an increased cell yield, as compared to a digestion in saline. This method required a much longer period of digestion than that described for other tissues, since skin is more difficult to dissociate than embryonic tissues or most other organs. A number of different digestion conditions was explored (Table I). Digestion could be conducted at room temperature for 24 hr, or at 37° for 6 hr. Digestion at room temperature for only 6 hr resulted in a lower cell yield, whereas cells were probably killed by digestion at 37° for 24 hr. The volumes of the digestion mixtures and the numbers of vessels used for the initial cell cultures were also important (Table II). Separation of the minced foreskin into three or four aliquots of trypsin mix before digestion resulted in lower cell yields. In addition the cells obtained after digestion grew best if initially dispersed into one or two culture vessels; further dilution of the cells led to a lower plating efficiency. The use of the more com-

TABLE II. INFLUENCE OF DIGESTION AND MEDIUM VOLUMES ON CELL YIELDS OF FIBROBLAST CULTURES ESTABLISHED FROM FORESKINS.^a

A		B	Cell yield (No./foreskin)
Volume of trypsin mix used per foreskin (ml)		Number of culture vessels used for initial plating	
A.	40	4	7.6×10^5
	40	2	5.4×10^6
	40	1	4.2×10^6
B.	40	2	3.4×10^6
	4×10	2	3.0×10^5
C.	40	2	1.8×10^6
	2×20	2	1.7×10^6
	4×10	2	4.7×10^4

^a For each experiment minced tissues from the foreskins were pooled and aliquots dispensed into the trypsin mix as indicated in column A. Cells were collected from each digestion as indicated in the methods section and initially plated in varying numbers of vessels as indicated in column B. Culture vessels were 50 cm² and contained 10 ml of medium. Cultures were harvested when the fullest was half confluent, and cell number determined in a hemacytometer.

TABLE I. TRYPSIN DIGESTION OF HUMAN FORESKINS FOR ESTABLISHMENT OF FIBROBLAST CULTURES.^a

Time (hr)	Temperature (°C)	Cell yield (No./foreskin)
6	22	3.4×10^5
24	22	1.0×10^6
6	37	2.2×10^6
24	37	$<1 \times 10^4$

^a The minced tissues from four foreskins was pooled and divided into four parts. Each part was digested for the indicated time and at the indicated temperature in 40 ml of trypsin solution and cultures established as described in Methods. Cultures were harvested when the fullest culture was half confluent, and cell number determined using a hemacytometer.

plete F-12 medium for the initial plating also appeared to increase the cell yield.

This method for establishing human foreskin cultures has consistently yielded viable cultures. The method has the advantage of yielding a large number of cells with one simple digestion, and it eliminates the difficult requirement of adherence of the minced pieces of tissue to the culture vessel. The cultures that result from multiple digestion appear similar in growth characteristics; however, like all fibroblasts cultures, there is undoubtedly a mixture of cell types present. Cultures obtained by this method have been used successfully for studies of lipid (7) and collagen (8) metabolism, and the method should be applicable for any system requiring human diploid fibroblast cell cultures.

Summary. A method is described for the establishment of diploid fibroblast cell cultures from human foreskins. Foreskins are minced and digested for a relatively long period of time in standard tissue culture medium containing trypsin and antibiotics. Undigested tissue is removed by filtration, and the cells are isolated by centrifugation

and dispensed in culture vessels. This procedure circumvents the explanation of large amounts of minced tissue fragments and yields larger numbers of cells in a short period of time.

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The Search for Submammalian Gastrins: The Identification of Amphibian Gastrin¹ (39529)

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Little information is available as to the phylogenetic distribution of the gastrointestinal hormone gastrin. It is hoped that by studying the phylogenetic evolution of this hormone one will be able to provide some insight into the development of hormone heterogeneity. Gastrin-like materials have been previously reported in nonmammalian species such as molluscs (1), elasmobranchs (2), reptiles, and birds (3, 4). This study represents the initial finding and characterization of the molecular heterogeneity of immunoreactive gastrin activity in the order Amphibia.

Methods. The gastrointestinal tracts (including liver and pancreas) of 30 bullfrogs (*Rana catesbeiana*), 10 *R. pipiens*, and 8 *Necturus* were dissected out quickly after the animals were killed by decapitation. The gastrointestinal tracts were sectioned into esophageal, fundic, antral, duodenal, midgut, and terminal intestinal components. The muscularis of each of these tissues was dissected away as much as possible in a petri dish in oxygenated amphibian Ringer's solution at pH 7.4. The mucosa of each of the above tissues as well as the liver and pancreas were then weighed and separately homogenized. The homogenates were then diluted in 20 ml of H₂O and boiled for 15 min. The solutions were allowed to cool for 10 min and then centrifuged and the supernatant solutions quantitated and stored at -20° until they were assayed for gastrin content.

Samples of blood and tissue extracts were fractionated by gel chromatography on Sephadex G-50 superfine columns (1 × 100 cm) using 0.02 M sodium barbital, pH 8.4,

containing 0.2 g/liter of sodium azide as the elution buffer. Each sample (1 ml) was applied to the column with approximately 1000 cpm of monoiodinated, ¹²⁵I-labeled G-17 and 2000 cpm of ¹³¹I (to indicate salt peak). The flow rates of the columns were adjusted to 6 ml/hr with fraction volumes of 1 ml.

Calibration of the Sephadex columns was accomplished using pure human G-13, G-17, and G-34 gastrins.⁴ In the situation where tissue samples were fractionated, 10 µl of human serum albumin was added to the sample prior to chromatography to provide a protein marker.

Gastrin content was determined via radioimmunoassay. Each tissue or column eluate sample was assayed in duplicate and the data expressed in terms of synthetic human gastrin 17-I equivalents in picograms (pg) per milliliter.

Briefly, the procedure for the radioimmunoassay is as follows: One-hundred microliters of sample (at various dilutions) was incubated at 4° for 72 hr in a mixture consisting of 900 µl of Veronal buffer (pH 8.4), 800 µl of monoiodinated synthetic human gastrin 17-I⁵ in buffer (~2000 cpm/tube), and 200 µl of antibody (Ab 1296, 1:1,000,000 final dilution) specific for the C-terminal region of G-17 (5).

At the end of 72 hr free gastrin was adsorbed onto Amberlite IRP-58M resin and separated from the antibody-bound fraction by centrifugation and decantation. Both free and antibody-bound fractions were counted in a gamma scintillation counter and the results are expressed as B/F ratio. The sensitivity of this assay was found to be 1 pg/ml.

Results. All three species of Amphibia

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⁴ Obtained courtesy of the Center for Ulcer Research and Education (CURE), Los Angeles, Calif.

⁵ Kindly supplied by Schwarz/Mann Pharmaceuticals, Orangeburg, N.Y.

contained measurable quantities of like immunoreactivity (Table I). In species of *Rana* studied, the highest concentrations of gastrin immunoreactivity were found in the antrum, followed by the duodenum and pancreas, in that tissue content. *Necturus*, however, was found to have the largest concentration in immunoreactivity in the portion of the gut just distal to the pyloric sphincter, a location which would correspond to the antrum in mammals. The antrum and duodenum, in decreasing order, were also found to contain assayable quantities of gas-

trastin. *Necturus*, in addition to the G-34 peak, was found to possess gastrin immunoreactivity in smaller fragments, some of which corresponded to G-17 and G-13.

Duodenal mucosa extracts from *R. catesbeiana* and *Necturus* yielded substantial gastrin immunoreactivity in the eluted void volume of the columns (Fig. 4). *R. catesbeiana* was found also to possess substantial gastrin immunoreactivity in the elution region corresponding to G-34. *Necturus*, in addition to the void volume peak, was found to possess gastrin immunoreactivity which corresponded to G-34 and G-17 elution volumes.

Discussion. Considerable debate has occurred as to where the hormone gastrin first appears on the phylogenetic tree. Gastrin immunoreactivity or biological activity has been found in molluscan species (1), in elasmobranchs (2), and in mammals such as pig, dog, cat, rabbit, and man (6).

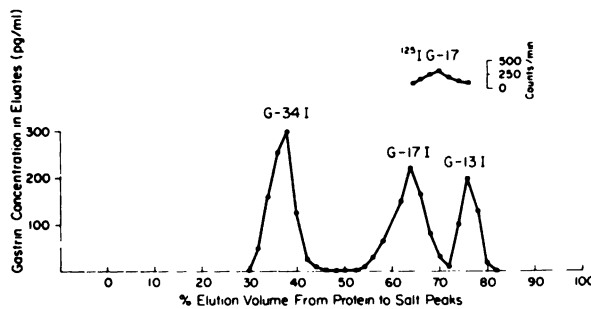
The current study found gastrin immunoreactivity to be present in the serum, antrum, duodenum, and pancreas of each amphibian species examined. Amphibians are monogastric and have a histologically distinct antrum (7). The sequence of tissue gastrin content was found to be similar to

Figure 1 shows the elution profile of the standard gastrins used to calibrate the elution columns. Fractionation of serum from *Rana catesbeiana* (Fig. 2) produced peaks of immunoreactivity corresponding to the void volume and the G-34 elution volume. The void volume peak may correspond to the "big big gastrin" (BBG) described in man (5) and was present in all samples chromatographed. Gastrin immunoreactive gastrin in antral mucosa from *Rana catesbeiana* (Fig. 3) was found to correspond primarily to G-34-I, with a small amount of immunoreactivity present as

TABLE I. THE TISSUE CONTENT OF IMMUNOREACTIVE GASTRIN ACTIVITY IN THREE SPECIES OF AMPHIBIA.

Species	Number of Animals	Gastrin content (ng/g \pm SE)				
		Serum	Antrum	Duodenum	Pancreas	Remaining gut
<i>R. catesbeiana</i>	30	33.71 \pm 19.43	40.22 \pm 13.3	5.22 \pm 1.36	0.62 \pm 0.12	0
<i>Ambystoma</i>	10	—	10.34 \pm 5.24	3.67 \pm 3.14	0.41 \pm 0.20	0
	8	—	2.27 \pm 0.62	14.04 \pm 2.83	0.13 \pm 0.01	0

Results are reported as nanograms of gastrin activity, expressed in synthetic human gastrin I equivalents, wet tissue weight.



Elution profile of standard human gastrins after filtration on Sephadex G-50 (superfine). Percentage volume is measured from protein peak (0%) to the salt peak (100%).

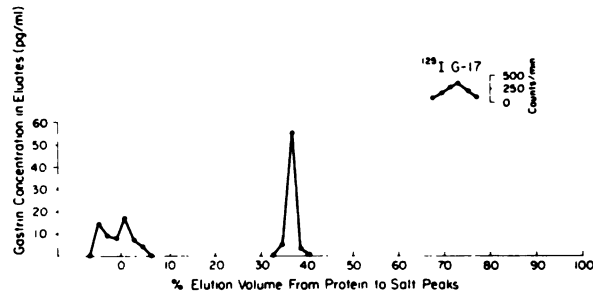


FIG. 2. Sephadex G-50 (superfine) gel filtration of immunoreactive gastrin in the blood (serum) of *Rana catesbeiana*.

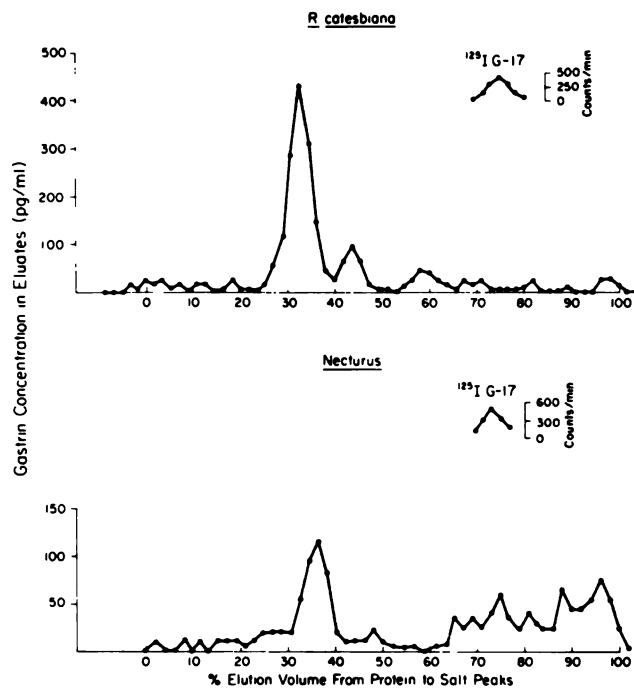


FIG. 3. Sephadex G-50 (superfine) elution profile of antral mucosa extracts from *R. catesbeiana* (top panel) and *Necturus* (lower panel).

that previously described in the cat, dog and man; viz., the antrum contained the largest concentration of gastrin, followed by the duodenum and pancreas. *Necturus*, however, was found to be an exception in that the duodenum was found to contain the largest tissue concentration of gastric activity, followed by the antrum and then pancreas.

Sephadex-gel chromatography of the immunoreactive gastrin in the blood and tissues of these amphibian species demon-

strated molecular heterogeneity. Gastrin molecular heterogeneity has previously been demonstrated in both the plasma and tissues of mammals (8).

In both the serum and duodenal extracts of *R. catesbeiana* and *Necturus*, a considerable portion of the gastrin immunoreactivity was found to be present in the void volume of the Sephadex G-50 column. This component of gastrin immunoreactivity has been termed "big, big gastrin" (BBG) and has been identified in both serum and duodenal

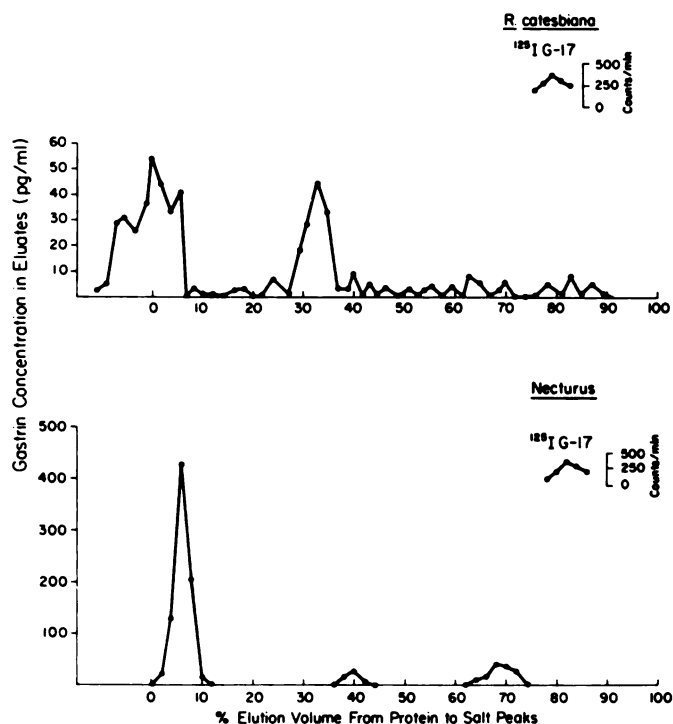


Fig. 2. Sephadex G-50 (superfine) elution profile of immunoreactive components from duodenal mucosa from *R. catesbeiana* (top) and *Necturus* (bottom).

s of humans (9). The origin and bioactivity of this immunoreactive component are yet unknown. It has been shown (9) that the fraction of the gastrin reactivity present in the BBG forms as one goes distally down the gastrointestinal tract, from being almost absent in the antrum to comprising a substantial portion of the gastrin immunoreactivity of the proximal jejunum. This same pattern is found to be present in *Necturus* and *R. catesbeiana*.

The predominant gastrin immunoreactivity in the sera and antral tissue extracts of *R. catesbeiana* was found in the elution volume corresponding to "big gastrin," the non-acid form of approximately 3900 daltons (10). The heptadecapeptide (G-17) form of gastrin which has been found in man could not be identified in *R. catesbeiana* sera.

Gastrin immunoreactivity corresponding to the G-17 molecular form was identified in the antral and duodenal tissue extracts of *R. catesbeiana*. The G-17 component was

found to comprise a significant portion of the antral gastrin immunoreactivity. Gastrin immunoreactivity corresponding to G-17 could be demonstrated in the antral tissue extracts of *Necturus*, with an additional portion of the gastrin immunoreactivity being present in elution volumes corresponding to the G-13 molecular form and possibly smaller fragments. Only trace quantities of G-17 immunoreactivity could be located in *Necturus* duodenal extracts.

The finding that gastrin immunoreactivity can be detected in Amphibia with an antibody with COOH-terminal specificity indicates that a certain similarity in the structure or amino acid sequence exists between this portion of amphibian gastrins and those of mammalian species. In addition, the observation that amphibian gastrins can be segregated into molecular species with molecular weights similar to those of mammalian gastrins lends some support to the hypothesis that this hormone in both species probably evolved from a similar precursor.

Gastrin has been found to be an effective

stimulus for gastric H^+ secretion in the bullfrog (11), and we presume that the endogenous gastrin activity we are describing serves as a physiological stimulus for H^+ secretion in amphibia.

Summary. Extracts of the digestive tracts of three amphibian species were examined by radioimmunoassay for gastrin-like materials. Gastrin was detected in all three species of amphibia examined. In *R. catesbeiana* and *R. pipiens* the largest tissue concentrations of gastrin were found in the antrum, followed by the duodenum and pancreas, respectively, in order of decreasing gastrin content. In the urodele *Necturus* the duodenum contained the highest tissue content of gastrin followed by the antrum and then the pancreas.

Molecular heterogeneity of gastrin was found in the sera of *R. catesbeiana* and in tissue extracts of *R. catesbeiana* and *Necturus*.

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Venoarterial Pathway for Uterine-Induced Luteolysis in Cows¹ (39530)

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cow, as in the ewe, there exists a uteroovarian pathway for uterine-induced luteolysis. Removal of one uterine horn in luteal maintenance when the corpus luteum (CL) is on the unilaterally hysterectomized side, but not when the CL is on the uterine-intact side (1, 2). Similarly, removal of the broad ligament between the uterine horn and adjacent ovary also results in luteal regression when a CL exists in the uterine horn (3). In addition, the luteolytic agent oxytocin (1), progesterone (4), and the uterine plastic coil (5) have been shown to be locally mediated, acting directly on the uterine horn and adjacent ovary.

In the ewe, the local pathway by which luteolysin passes between the uterine horn and adjacent ovary is venoarterial, involving veins which drain the uterine horn and the ipsilateral ovarian vein (6). Although the mechanism by which luteolysin passes from vein into artery is unresolved, passage is likely facilitated by the close contact between veins and the ovarian artery. The ovarian artery is tortuous and closely applied to the surface of the uteroovarian (ovarian) vein. The local uteroovarian venoarterial pathway for uterine-induced luteolysis is demonstrated by experiments in which surgical anastomosis of vessels in the uteroovarian vascular pedicle (8, 9, 10). In the cow, hysterectomy adjacent to the uterine horn during the CL resulted in luteal regression, however, surgical anastomosis

of either the main uterine vein or the ovarian branch of the ovarian artery from the intact side to the corresponding vessel on the hysterectomized side resulted in luteal regression on the hysterectomized side. Results therefore demonstrated, that in the ewe, a local venoarterial pathway (involving main uterine vein and adjacent ovarian artery) operated in the physiologic process of uterine-induced luteal regression.

In the cow, a common uteroovarian (ovarian) vein drains the ovary, uterine tube and much of the uterine horn and the ovarian artery is very tortuous and closely applied to the surface of the uteroovarian vein (11, 12; Fig. 1). The similarity of vascular anatomy of the uteroovarian pedicle in the ewe and cow therefore, led to the hypothesis that the local uteroovarian pathway for uterine-induced luteolysis in the cow is also venoarterial in nature (11). The purpose of the present experiment was to determine whether the main uterine vein and ovarian artery were involved in the local uteroovarian luteolytic pathway in cows.

Materials and Methods. Mature Holstein cows were observed twice daily for estrus and the day that a cow stood for mounting was designated day 0 of the estrous cycle. Ovulation and CL formation and development were confirmed by rectal palpation. Cows were fasted for 48 hr and surgery was done on days 8, 9, 10 or 11 of the cycle. Anesthesia was maintained with halothane. Mid-ventral laparotomy was done and CL were marked in all cows with India Ink. After surgery, cows were observed twice daily for estrus and were necropsied on day 30 (9 days after the expected return to estrus).

Cows were allotted to groups 1-4 at surgery and group 5 was added at necropsy (Fig. 2). In all cows a unilateral hysterectomy was done (contralateral to CL in group 1 and ipsilateral to CL in all other groups) and the cut edge of the broad ligament on

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²Graduate Trainees, Endocrinology-Reproductive Physiology Training Program, University of Wisconsin. R. J. Mapletoft is supported by the Natural Sciences and Engineering Research Council of Canada.

³Reprinted in part from a paper presented here for illustrative purposes and was published in *Am. J. Vet. Res.* 35, 193 (1974).

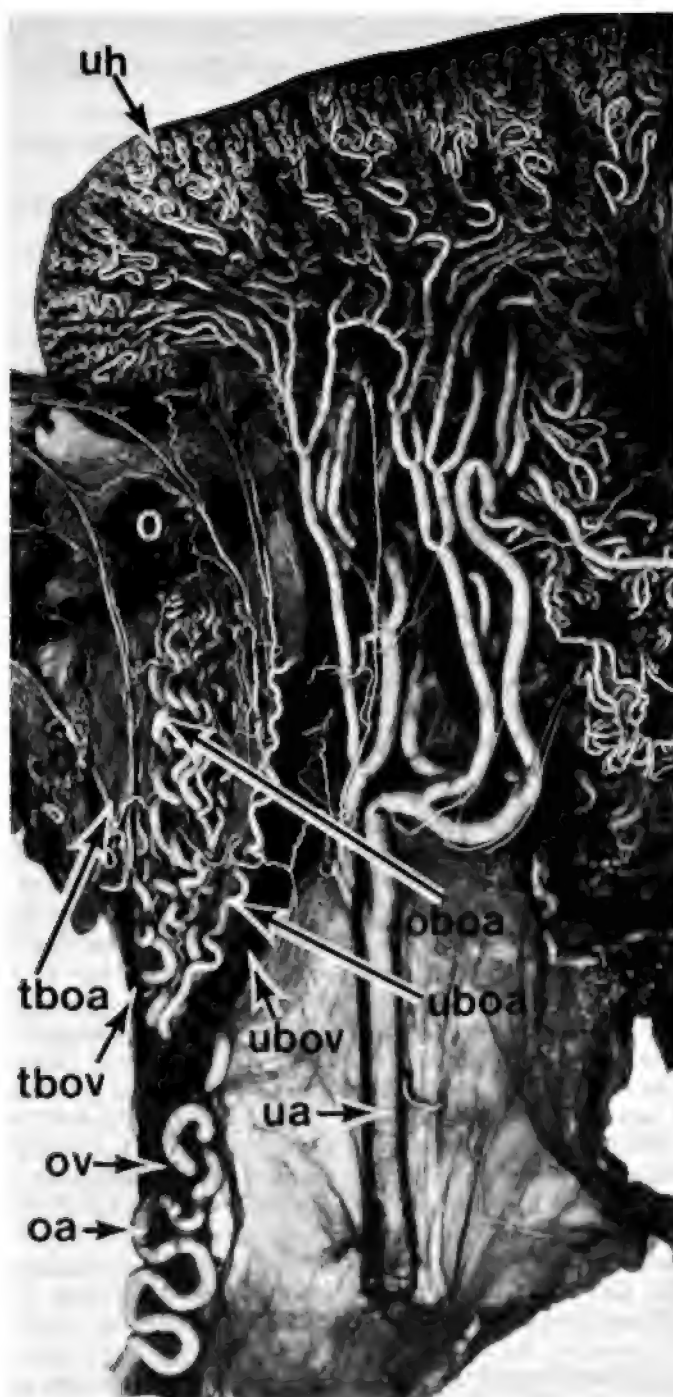


FIG. 1. Dorsal view of the left uteroovarian vascular pedicle in a heifer. Veins (dark) and arteries (light) injected and tissues were fixed and cleared. A common uteroovarian (ovarian) vein drains the ovary, uterine and much of the uterine horn. The ovarian artery is very tortuous and is closely applied to the uteroovarian vein. Terminally, the ovarian artery divides into a uterine branch and an ovarian branch and the latter divides into tubal branch and numerous ovarian branches which enter the ovary. It is proposed that the local luteolytic effect of the uterus in cows is exerted through a venoarterial pathway which involves discharge of the uterine luteolysin into the uteroovarian veins which drain the uterine horn (main uterine vein and uteroovarian vein) and transfer into the adjacent ovarian artery which carries the luteolysin to the ovary. O = ovary, uh = uterine horn, oa = ovarian artery; ov = ovarian (uteroovarian) vein; ua = uterine artery; oboa = ovarian branches of ovarian artery; tboa = tubal branch of ovarian artery; tbov = tubal branch of ovarian vein; ubov = uterine branch of ovarian vein; uboa = uterine branch of ovarian artery.

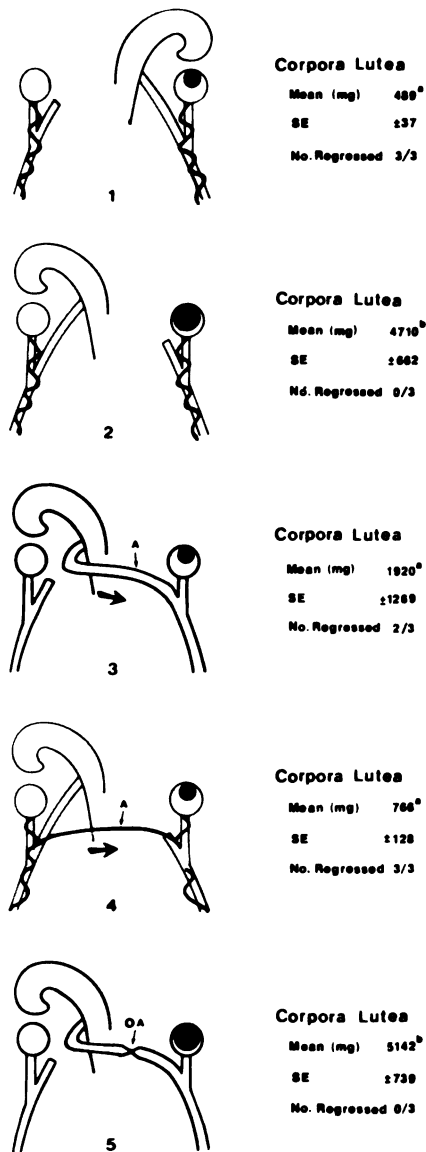


FIG. 2. Effect on the corpus luteum in cows of surgical anastomosis (A) of the main uterine vein (group 3) or the uterine branch of the ovarian artery (group 4) from uterine-intact side to hysterectomized side. The venous anastomosis in group 5 was occluded (OA). Surgery was done on days 8–11 of diestrus and necropsies were done on day 30. All cows were unilaterally hysterectomized, ipsilateral (group 1), and contralateral (groups 2–5) to the CL and surgical anastomosis of the main uterine vein (groups 3 and 5) and of uterine branch of the ovarian artery (group 4) was performed. The arrow in groups 3 and 4 indicates the direction of blood flow. Mean CL weights with different superscripts are significantly different ($P < 0.05$).

the hysterectomized side was sutured to the intact uterine horn, as described (1). In group 3, the main uterine vein (uterine branch of the ovarian vein) on the uterine-intact side was surgically anastomosed to the corresponding vein on the hysterectomized side. An end-to-end venous anastomosis was done with 6-0 cardiovascular silk using standard vascular surgical techniques (8). Patency of anastomosis was checked by observation for free flow and the adjacent serosa was sutured over the anastomosis to give it additional support. In group 4, the uterine branch of the ovarian artery on the uterine-intact side (donor artery) was surgically anastomosed to the uterine branch of the ovarian artery on the hysterectomized side (recipient artery). The technique for freeing the arteries and performing an end-to-end anastomosis has been described (9). Upon completion of the anastomosis, the ovarian artery on the recipient side was ligated 3 times at levels approaching the aorta to minimize the development of collateral vessels so that the ovary on that side would be supplied only through the surgical anastomosis. Patency of the surgical anastomosis was indicated by distension of the recipient artery and maintenance of color of the CL in the recipient ovary. Sodium heparin solution was given iv (40,000 units) to cows in groups 3 and 4 immediately after removal of the uterine horn and every 12 hr for 36 hr, beginning immediately after surgery. Penicillin and streptomycin were given twice daily for 3 days.

At necropsy on day 30, marked CL were removed, weighed and classified as regressed (small, white, firm and encapsulated), maintained (large, soft and flesh colored), or partially regressed (intermediate size, color and consistency). In addition, ovaries were examined for follicular development and evidence of recent ovulations. The surgically anastomosed vessels were examined for patency by gently flushing saline into the uterine artery (group 3) or ovarian artery (group 4) on the donor side. Patency was indicated by venous discharge of saline on the recipient side in group 3 and oozing of saline from the site of the removed CL in group 4. Cows in group 3 with an occluded venous anastomosis were replaced and allot-

ted to group 5, whereas cows in group 4 with an occluded arterial anastomosis were replaced.

Weights of CL were statistically analyzed by a one-way analysis of variance and mean CL weights were compared by the protected lsd test for multiple comparisons (significant F value for treatment effect in the analysis of variance).

Results. At necropsy, 3 cows had an occluded venous anastomosis and 1 cow had an occluded arterial anastomosis. Based on multiple comparisons (Fig. 2), mean weight of CL was less ($P < 0.05$) in group 1 controls with unilateral hysterectomy contralateral to CL (489 mg; CL regressed in 3 of 3 cows), in group 3 with unilateral hysterectomy ipsilateral to the CL and surgical anastomosis of the main uterine vein from intact side to hysterectomized side (1920 mg; CL regressed in 2 of 3 cows) and in group 4 with unilateral hysterectomy ipsilateral to CL and surgical anastomosis of uterine branch of ovarian artery from intact to hysterectomized side (708 mg; CL regressed in 3 of 3 cows) than in group 2 controls with unilateral hysterectomy ipsilateral to CL (4710 mg; CL maintained in 3 of 3 cows) and in group 5 with unilateral hysterectomy ipsilateral to CL and occluded venous anastomosis (5142 mg; CL maintained in 3 of 3 cows). The mean weight of CL was not significantly different among groups 1, 3 and 4 or between groups 2 and 5.

Discussion. Removal of the uterine horn on the side opposite to the ovary bearing the CL appeared to have no effect on the degree of luteal regression by day 30 suggesting that surgical operation on days 8 to 11 of diestrus did not in itself interfere with luteal regression. This was not, however, tested critically, since unoperated controls were not included. Removal of the uterine horn on the side ipsilateral to the CL resulted in luteal maintenance confirming the involvement of a local or unilateral uteroovarian pathway for uterine-induced luteolysis in cows.

Surgical anastomosis of the main uterine vein from intact side to hysterectomized side (Fig. 2, group 3) resulted in luteal regression in 2 of 3 cows indicating that the uterine luteolysin was being delivered to the

ovarian vascular pedicle on the hysterectomized side. In the cow in which luteal regression did not occur it was noted at necropsy that the surgically anastomosed veins were twisted by tension on the broad ligament. Saline could only be flushed through the surgical anastomosis when the serosa over the veins was dissected away. Cows in group 5 (occluded venous anastomosis with thrombus formation) provide additional support for the hypothesis that the main uterine vein serves as the uterine or initial component of the pathway. Luteal maintenance occurred in 3 of 3 cows indicating that a functional (patent) vein is necessary to deliver the luteolysin to the site of venoarterial transfer (presumably in the ovarian vascular pedicle).

When the ovarian arterial blood from the uterine-intact side supplied the CL on the unilaterally hysterectomized side luteal regression occurred (Fig. 2, group 4) indicating that the ovarian artery delivered the uterine luteolysin to the ovary through the surgical anastomosis. This result indicates that the ovarian artery serves as the distal or ovarian component of the local uteroovarian pathway for uterine-induced luteolysis in cows. Presumably, venoarterial transfer of uterine luteolysin occurred on the uterine-intact side in areas of close contact between veins containing uterine venous blood and the ovarian artery. The uterine branch of the ovarian artery was used for the surgical anastomosis because the ovarian branches of the ovarian artery were too small and too inaccessible for surgical anastomosis. Ligations of the proximal segment of the recipient ovarian artery facilitated flow through the anastomosis and minimized the probability that the recipient ovary would be supplied by collateral vessels which would contain no luteolysin. Indeed, upon completion of the surgical anastomosis and ligations on the recipient side, the vessels distended with blood and the CL maintained its pink color, indicating that it was being supplied by arterial blood.

In cattle there exists a prominent uteroovarian arterial anastomosis between a branch of the uterine artery and the uterine branch of the ovarian artery (11, 12). It appeared to be quite dynamic, being significantly

prominent on the side of the CL and more apparently changes in diameter, depending on the side of ovulation (11). Physiologic role of the difference between the two sides in the diameter of the anastomosis and the direction of blood flow after anastomosis are not known. However, it has been suggested that the uteroovarian arterial anastomosis is necessary for normal cyclic ovarian function (12, 13). Local anastomosis of the main uterine vein from intact to hysterectomized side resulted in luteal regression on the hysterectomized side. This result tends to rule out the necessity of the uteroovarian arterial anastomosis for luteal regression in the cow (2).

The cow in which the arterial anastomosis became occluded, partial luteal regression occurred (2219 mg). Perhaps a reduced ovarian arterial blood flow can result in at least partial luteal regression in cows, as has been observed in the ewe (10). This could be a suggested mode of luteal regression in group 4, however, available evidence tends to rule this possibility out in ewes in which the blood flow to the CL had been compromised, not only CL regression but also ovarian inactivity and atrophy occurred (14). Luteal regression occurred in group 4 cows with no apparent reduction in ovarian activity. Although a local anastomosis was not done in control (group 2), transection and reanastomosis of the ovarian artery in unilaterally hysterectomized ewes did not cause any evidence of luteal regression (10). Furthermore, the ovary on the recipient side in group 4 cows was not deprived of arterial blood during the anastomosis procedure. If the proximal segment of the recipient uterine artery was ligated, the CL maintained its pink color indicating that it continued to receive arterial blood (presumably through the surgical anastomosis). Present results in the cow are therefore consistent with those reported previously in the ewe (10). Although estrous cycle length was analyzed, there did not appear to be any difference between cows with surgical anastomosis (groups 3 and 4) and group 1 controls. Obviously, studies are required to determine critically the adequacy of the veno-

arterial pathway for complete luteolysis.

Results, therefore, indicate that the main uterine vein serves as an initial or uterine component and that the ovarian artery serves as a distal or ovarian component of the local uteroovarian pathway for uterine-induced luteolysis in the cow. Results are consistent with the hypothesis that the uterus regulates the lifespan of the CL in cows through a local venoarterial pathway.

Summary. The involvement of the main uterine vein and ovarian artery in the local pathway for uterine-induced luteolysis was studied in mature Holstein cows. At surgery on days 8–11 of diestrus, all cows were unilaterally hysterectomized and allocated to one of 4 groups: 1) controls with hysterectomy contralateral to CL, 2) controls with hysterectomy ipsilateral to CL, 3) hysterectomy ipsilateral to CL and surgical anastomosis of the main uterine vein from intact to hysterectomized side, and 4) hysterectomy ipsilateral to CL and surgical anastomosis of ovarian artery from intact to hysterectomized side. At necropsy on day 30, the surgical anastomosis was examined for patency and CL were weighed. Cows with an occluded venous anastomosis were reassigned to group 5. CL weights from 15 cows (3 per group) were analyzed. Mean weight of CL was less ($P < 0.05$) for group 1 controls (489 mg), group 3 with venous anastomosis (1920 mg), and group 4 with arterial anastomosis (708 mg), than for group 2 controls (4710 mg) or group 5 with occluded venous anastomosis (5142 mg). Mean weight of CL was not significantly different among groups 1, 3, and 4 or between groups 2 and 5. Results indicate the involvement of a venoarterial pathway in the local uteroovarian luteolytic effect in cows. The main uterine vein serves as the proximal or uterine component and the ovarian artery serves as the distal or ovarian component of the pathway.

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Absorption of Selenite and Selenomethionine from Ligated Digestive Tract Segments in Rats¹ (39531)

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ted information is available on the
ion of selenium from the digestive
animals. By use of a nonabsorbable
and radioactive selenium, this ele-
as shown to be secreted into the first
h and absorbed from the remainder
small intestine in sheep and swine (1).
tion of this element did not occur
ie rumen of sheep or from the stom-
swine. *In vitro* work with everted
al sacs from the golden hamster re-
that selenomethionine was actively
rted, but selenite and selenocystine
ot (2). Since ouabain inhibited the
rt of selenomethionine, the authors
ed that the sodium pump may play a
the transport of this amino acid (3).
nsport of selenomethionine was in-
by the corresponding sulfur analog,
onine, but transport of selenite and
ystine was not inhibited by sulfite
tine, respectively.

ligated segment technique has been
study the absorption of copper, zinc,
enum, and iron from various parts of
estive tract of rats (4). Copper was
o be absorbed most readily from the
1 whereas zinc and iron were taken
t rapidly from the duodenum. In con-
olybdenum appeared to be absorbed
it equal rates from the duodenum,
ion, or ileum. Since this technique
he advantage of studying the absorp-
m various parts of the digestive tract

in the intact animal, it was used in the pres-
ent study to investigate the absorption of
selenium. Two chemical forms of selenium,
selenite and selenomethionine, were tested
to compare their absorption patterns.

Materials and methods. Mature OSU
brown rats, about 1 year old, from our
breeding colony were used in these studies.
Most of the female rats had produced three
litters of young, and the males had been
used for breeding purposes. The rats had
been fed solely Purina rat chow since wean-
ing. This chow contained about 0.3 ppm of
selenium.

The absorption of selenium from the li-
gated segments of rats was studied essen-
tially by the technique reported by Van
Campen and Mitchell (4). After an over-
night fast, the rats were anesthetized with
ether and the peritoneal cavity was opened
by a 3.0- to 4.0-cm midline incision. Except
for the stomach, the segments to be studied
were displaced, still intact, from the abdom-
inal cavity and ligated anteriorly and poste-
riorly. The latter ligature was left loose, the
needle was inserted through the intestinal
wall, the ligature was tightened around the
needle, and radioactive selenite or seleno-
methionine was injected into the lumen.
The segments studied were the stomach, the
duodenum (about 0.5 to 7.5 cm distal to the
pylorus), the jejunum (about 32 to 40 cm
proximal to the ileocecal junction), and the
ileum (about 2 to 8 cm proximal to the
ileocecal junction). The stomach was ligated
only at the pylorus, leaving the esophagus
open. After the injection had been made,
the segment was returned to the body cavity
and the incision closed with sutures and
stainless-steel wound clips before returning
the rats to their cages. After 3 hr, the rats
were killed by anesthesia with ether, blood
was taken by heart puncture with needle
and syringe, and tissues (heart, liver, and
kidney) were taken for selenium-75 count-

ished with the approval of the Director of the
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ent address: Analytical Chemical Division,
Department of Agriculture, Salem, Oreg.

ent address: Department of Fisheries and
University of Washington, Seattle, Wash.

ing in a Packard Tri-Carb, Model 3002, gamma counter.

The heart and kidney were placed in saline, blotted with tissue paper, and placed in counting tubes. The liver was also placed in saline, blotted with tissue paper, and weighed. A sample was placed in a tared counting tube. The data are expressed as percentage of the administered activity recovered in each tissue. Values for blood were calculated by multiplying the counts per minute per gram of blood by 0.07 times the body weight of the animals, and values for kidneys were obtained by doubling the counts obtained from one kidney. The data were subjected to an analysis of variance for statistical evaluation.

About 5 μCi (0.5 μCi of $^{75}\text{Se}/\mu\text{g}$ of selenite) as sodium selenite in 0.3 ml of saline or 5 μCi (10 μCi of $^{75}\text{Se}/\mu\text{g}$ of selenomethionine) as L-selenomethionine in 0.3 ml of saline were injected into each ligated segment. These isotopes were obtained from Amersham/Searle Corporation, Arlington Heights, Ill. The purity of these compounds was not determined.

Results. Essentially no selenium as sodium selenite was absorbed from the stomach (fig. 1). However, extensive absorption occurred from the other segments. Slightly greater amounts of selenium were found in liver when the selenium was placed in the duodenum than when placed in the jejunum or ileum. The liver contained significantly ($P < 0.01$) more selenium than the blood. Of the tissues examined, the heart contained the smallest amount, followed by the blood, with the liver containing the greatest amount of the isotope. The combined data from the duodenum, jejunum, and ileum revealed that the liver contained about 10 times more radioactivity per gram than the blood on a per milliliter basis.

As was the case with selenite, there was very little absorption of selenomethionine from the stomach and extensive absorption from the other segments (Fig. 2). The whole liver, however, contained amounts of selenium similar to that found in the blood, in contrast to that observed for selenite. The combined data from the duodenum, jejunum, and ileum revealed that the liver contained about 3.5 times more radioactiv-

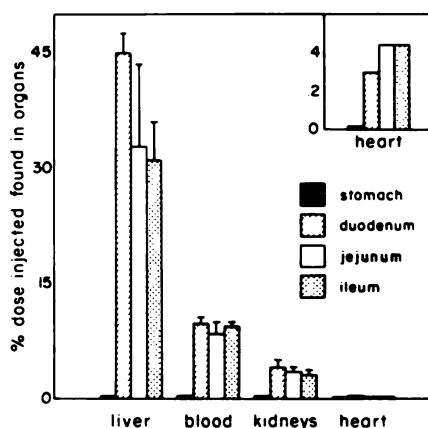


FIG. 1. Absorption of $[^{75}\text{Se}]$ selenite from ligated intestinal segments in rats. The values represent the means + standard deviation of five rats (three males and two females) for each intestinal segment. The average weight of the males was 468 g and the average weight of the female rats was 286 g. The males and females showed similar patterns of absorption.

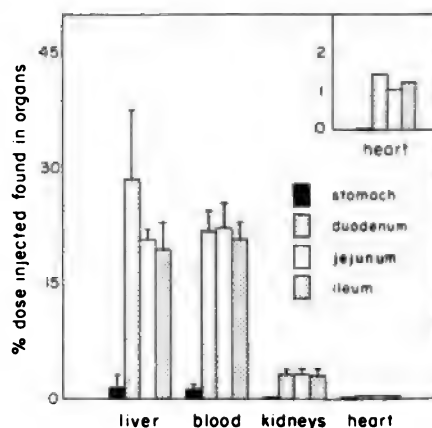


FIG. 2. Absorption of $[^{75}\text{Se}]$ selenomethionine from ligated intestinal segments in rats. The values represent the means + standard deviation of five rats (two males and three females) for each intestinal segment. The average weight of the males was 420 g and the average weight of the female rats was 261 g. The males and females showed similar absorption patterns.

ity on a per gram basis than the blood on a per milliliter basis. On the basis of percentage of dose in each tissue, the heart contained the least amount, followed by the kidneys, but the blood and liver contained about an equal percentage of the isotopic dose.

On the basis of the summed recovery from liver, blood, kidneys, and heart there was

ference between the absorption of selenium and selenomethionine from the four intestinal tract segments (Fig. 3). Figure 3 actually illustrates the minimal absorption of both selenium-containing compounds from the stomach, and absorption is significantly less ($p < 0.001$) than from the three segments. The greatest absorption for both selenium-containing compounds appears to be from the duodenum, nonsignificantly less absorption from jejunum or ileum.

Discussion. Whether selenium was in the form of selenite or selenomethionine, essentially no absorption occurred from the stomach. Our data are in agreement with those of Leeper and swine, which indicate no absorption of selenium from the preintestinal (1). The significance of these observations is that the various elements are not absorbed from the same intestinal segment. Essentially no absorption occurred from the stomach, which is in marked contrast to that reported for copper (4). Copper was reported to be absorbed to the greatest extent from the stomach. Interestingly, the chemical form of selenium used in the present study had no significant influence on its absorption from the intestinal segments, but the ratio (10.4:1) of the concentration of selenium in liver on a per gram basis to that in the blood on a per milliliter basis was much greater than this ratio (10:1) for selenomethionine.

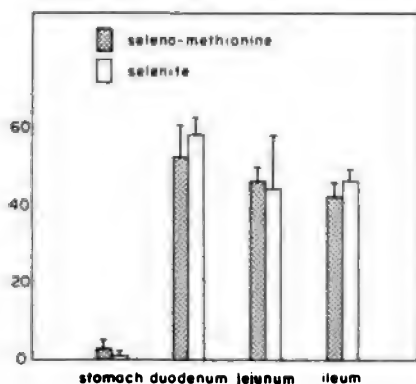


Fig. 3. Total absorption of $[^{75}\text{Se}]$ selenite or selenomethionine from ligated intestinal segments. The data are the accumulated values presented in Tables 1 and 2.

In vitro work with everted intestines from the golden hamster indicated that selenomethionine was actively transported across the intestinal tract, but selenocystine and selenite were not (2, 3). No explanation was offered by these workers for the difference in absorption of these selenium-containing compounds. It cannot be determined from the present data whether selenite and selenomethionine were actively transported. Since both compounds showed similar absorption patterns from the various intestinal tract segments, it might be suspected that these two selenium compounds were absorbed by similar mechanisms.

The ligated intestinal segment technique has been used to study the effects of zinc, cadmium, silver, and mercury on copper absorption (5), to study the interference of copper with zinc absorption (6), and to study the influence of amino acids on iron absorption (7). Thus, this technique would appear to be useful for studying various factors influencing selenium absorption, particularly the effects of sulfur analogs on absorption of this element. The *in vitro* inhibition of selenomethionine absorption by methionine in the everted intestinal sacs of the golden hamster (3) indicates that such studies should yield useful information.

Summary. The absorption of selenium as sodium selenite or as selenomethionine was studied with ligated intestinal segments in rats. Essentially no absorption of either compound occurred from the stomach, whereas the greatest absorption occurred from the duodenum, with slightly smaller amounts from the jejunum or ileum.

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Comparison of the Suppression of Interferon Production and Inhibition of Its Action by Vitamin A and Related Compounds (39532)

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We have previously shown that vitamin A inhibited the antiviral action of interferon (1) and that this inhibition seemed to be due to an effect of vitamin A on the interferon molecule (2). Inhibition of interferon action showed a critical dependence on the form of vitamin A that was tested.

We subsequently reported that retinoic acid (vitamin A acid) suppressed the production of interferon (3). The suppression of interferon production was shown not to be due to inactivation of interferon. For instance, retinoic acid treatment of cells was required only prior to interferon production for suppression. Also, suppression of production occurred in the presence of 10% calf serum (CS) whereas interferon inactivation by vitamin A did not. Inactivation did not occur in 10% CS, presumably because of a competition of the serum proteins for the vitamin A. These observations suggested that suppression of interferon production and inhibition of its action might be dependent on different parts of the vitamin A molecule. Therefore, we have investigated the structural requirements for vitamin A suppression of interferon production and inhibition of interferon action.

Cells. Mouse L-929 cells were maintained by weekly passage in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum (CS). This medium contained 125 μ g of streptomycin and 150 units of penicillin/ml.

Virus. A large-plaque variant of vesicular stomatitis virus (VSV), Indiana strain, and a lentogenic strain of Newcastle disease virus (NDV) were grown in the allantoic cavity of developing chick embryos. The pool of VSV contained about 1×10^9 plaque forming units (PFU)/ml when assayed on L-929 mouse cells. The pool of NDV contained about 640 hemagglutination units/ml.

Production and assay of interferon.

Mouse interferon was produced by infection of L-929 cells with a lentogenic strain of NDV. Supernatant fluids were harvested 24 hr after infection and dialyzed against pH 2 buffer for 5 days at 4° and then against Gey's balanced salt solution (BSS) to restore the pH to neutrality.

A plaque reduction assay using VSV and L-929 cells was employed for quantitating interferon. Confluent cell monolayers in 2-oz glass bottles were treated overnight with two fold serial dilutions of interferon preparations in triplicate or quadruplicate. Supernatant fluids were then aspirated and cells infected with 0.2 ml of a dilution of VSV containing about 300 PFU. After a 1-hr incubation at room temperature to permit adsorption of virus, each monolayer was overlaid with 5 ml of MEM containing 1% methyl cellulose, 5% calf serum, 25 mM Hepes buffer, 125 μ g/ml of streptomycin, and 250 units/ml of penicillin (overlay medium). The cultures were incubated for 48 hr at 37° and monolayers were then stained with crystal violet. Plaques were enumerated after $\times 6.5$ magnification of the monolayers by use of a photographic enlarger. The 50% plaque depressing dose (PDD₅₀) was defined as the amount of an interferon preparation, in microliters, that inhibited 50% of the plaques from developing as compared to the controls.

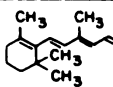
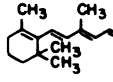
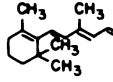
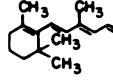
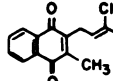
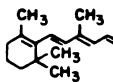
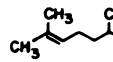
Chemicals. All *trans* forms of retinoic acid (vitamin A acid), retinol (vitamin A alcohol), retinyl acetate (acetate ester of vitamin A), and retinal (vitamin A aldehyde) were obtained from Sigma Chemical Company, St. Louis, Mo. Vitamin K₁ was obtained from Schwarz/Mann, Orangeburg, N.Y. *Trans*- β -carotene and citronellol were obtained from Aldrich Chemical Company, Milwaukee, Wis. Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific Company, Pittsburgh, Pa.

Stock solutions were made by dissolving each compound in DMSO. For experimental purposes, the compounds were made $6.7 \times 10^{-5} M$ in medium (equivalent to 20 $\mu\text{g}/\text{ml}$ of retinoic acid).

Results and discussion. Different forms of vitamin A and related compounds dissolved in DMSO were tested to determine their effect on the antiviral activity of interferon. Retinoic acid, retinol, retinal, retinyl acetate, vitamin K₁, β -carotene, and citronellol were each made $6.7 \times 10^{-5} M$ in an interferon preparation diluted 1 to 10 in MEM. Controls received an equivalent amount of DMSO (1%). After 24 hr at 37°,

the mixtures were further diluted 1 to 100 in MEM with 5% CS and assayed for residual interferon activity. Table I shows that retinoic acid and retinol were similarly effective at inhibiting interferon activity while retinal and retinyl acetate were considerably less inhibitory. These data suggested that the terminal group on the vitamin A molecule was important for the inhibitory effect on interferon activity. This concept is supported by the low level of inhibitory activity of β -carotene on interferon action. β -Carotene is a dimer of vitamin A with a ring on both ends of the molecule. Since β -carotene was not very inhibitory for interferon action, it

TABLE I. COMPARISON OF THE SUPPRESSION OF INTERFERON PRODUCTION AND INHIBITION OF ITS ACTION BY VITAMIN A AND RELATED COMPOUNDS.

Compound tested ^a	Percentage of suppression of interferon					
	Antiviral activity			Production		
	Replicate experiments ^b	Mean \pm SD	P	Replicate experiments	Mean \pm SD	P
 RETINOIC ACID	60,64,65,65	63 \pm 3	<0.001	75,67,61	68 \pm 7	<0.001
 RETINOL	57,100	79 \pm 21	<0.01	67,47	57 \pm 14	<0.01
 RETINAL	24,17	21 \pm 5	NS ^c	75,68	72 \pm 5	<0.001
 RETINYL ACETATE	14,0	7 \pm 10	NS	51,40	46 \pm 8	<0.01
 VITAMIN K ₁	2,0	1 \pm 1	NS	19,0	10 \pm 13	NS
 β -CAROTENE	29,24	27 \pm 4	<0.05	57,48	53 \pm 6	<0.01
 CITRONELLOL	0,0	0	NS	0,0	0	NS
DMSO (CONTROL)	0 ^d	0	—	0	0	—

^a Compounds were tested at $6.7 \times 10^{-5} M$ which is equivalent to 20 $\mu\text{g}/\text{ml}$ of retinoic acid.

^b Each value represents a separate interferon assay compared to a control interferon assay, both of which were performed in triplicate or quadruplicate. Cultures were challenged with about 300 PFU of VSV.

^c Statistically not significant (all P values >0.3). Statistical analysis was done by Student's *t* test.

^d By definition 0% suppression. DMSO had no effect on interferon action or production at the concentration employed (1%). Control interferon levels were from 8000 to 16,000 PDD₅₀ units/ml.

seems that the ring portion of the vitamin A molecule was not very important in the inhibition of interferon action. Furthermore, citronellol, an analog of the side chain of retinol, was not inhibitory, suggesting that the conjugated double bond system was also important in the interaction of vitamin A with the interferon molecule. Another fat-soluble vitamin, vitamin K₁, did not inhibit interferon activity.

The same compounds were tested for their effect on interferon production. NDV was adsorbed to L-929 cells for 1 hr at room temperature. Cell cultures were then washed and received either retinoic acid, retinol, retinal, retinyl acetate, vitamin K₁, β -carotene, or citronellol at 6.7×10^{-5} M in MEM with 10% CS. Ten percent CS was employed because vitamin A does not inhibit interferon activity under these conditions (2). In contrast to the marked dependence on the form of vitamin A required for inhibition of interferon action, all forms of vitamin A tested suppressed interferon production (Table I). This observation suggested that the ring portion of the vitamin A molecule was of primary importance for the suppression of interferon production. That β -carotene, a dimer of vitamin A with a ring group at each end of the molecule, was suppressive for interferon production is consistent with this idea. Also, the lack of a suppression by citronellol, an analog of the side chain of retinol, supports this concept. Vitamin K₁ was also not very suppressive.

These results have illustrated that vitamin A can suppress both interferon action and production. The inhibitory effects, however, appear to result from different mechanisms. The inhibition of interferon activity by vitamin A seems to be due to an effect of vitamin A on the interferon molecule (2), while the suppressive effect on interferon

production is clearly due to an effect of vitamin A on the cell which has been induced to make interferon (3). It is interesting that the different mechanisms of action of vitamin A on interferon action and production apparently occur by different moieties of the vitamin A molecule (Table I). Inhibition of interferon action seems to be most dependent on the side chain of the vitamin A molecule. The side chain apparently requires a conjugated double bond system and a hydrophilic terminal group (hydroxy group for retinol and carboxy group for retinoic acid) to inhibit interferon action. In contrast, the ring portion of the vitamin A molecule seems to be most important for the suppression of interferon production.

Summary. We have investigated the structural requirements for vitamin A suppression of interferon production and inhibition of interferon action. Inhibition of interferon action seems to be most dependent on the side chain of the vitamin A molecule. The side chain apparently requires a conjugated double bond system and a hydrophilic terminal group to inhibit interferon action. In contrast, the ring portion of the vitamin A molecule seems to be most important for the suppression of interferon production.

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Antibody-Mediated Bacteriolysis: Enhanced Killing of Cyclacillin-Treated Bacteria (39533)

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variations in the susceptibility of a microorganism to various antibiotics have generally been attributed to differing effects of antibiotics on bacterial metabolism. Recent studies in this and other laboratories, have indeed shown significant differences in the susceptibility of a microorganism to a single antibiotic when tested *in vitro* and *in vivo*. For example, cyclacillin, a semisynthetic antibiotic, is much more effective against *Escherichia coli* *in vivo* than *in vitro* (1, 2). Previous studies in this laboratory suggested that this difference may be due to a synergistic effect of host factors present *in vivo* absent *in vitro* (3, 4). For example, *E. coli* incubated with sublethal doses of cyclacillin *in vitro* were more susceptible to phagocytosis by rodent leukocytes than untreated bacteria or bacteria treated with an antibiotic which had no effect on the organism. The present study was designed to determine the extent of influence of cyclacillin on host factors associated with specific lymphocyte activation i.e., to an antibody responsiveness *in vivo*. It was found that antibody-treated *Escherichia coli*, although not killed by a low dose of cyclacillin, nevertheless became highly susceptible to complement-dependent antibody-mediated bacteriolysis.

Materials and methods. *E. coli* strain 8, previously used for immunologic studies concerning responses at the single cell level, was passaged for 18 hr at 37° on heart infusion (BHI) agar slants (Wyeth Laboratories, Detroit, Mich.). For *in vitro* studies the bacteria were cultured overnight at 37° in 10 ml of BHI broth, harvested by centrifugation, and washed several times in sterile saline at 4°. The minimal bactericidal concentration (MIC) of cyclacillin was determined against reference standards, ampicillin and penicillin G, against the *E. coli* was determined by standard serial twofold dilution

assays. Antibiotic dilutions were prepared in 0.5-ml volumes of sterile BHI broth in 13 × 75-mm-diameter tubes closed with plastic stoppers, using a stock solution containing 100 µg of each antibiotic per milliliter of saline. For MIC determinations, 0.1 ml of a washed overnight culture of the *E. coli* (2×10^7 bacteria) was added to each antibiotic dilution and the tubes incubated at 37° for 12 to 18 hr. The antibacterial concentration was determined qualitatively by examining the tubes for visual growth and quantitatively by transferring 0.1 ml to 10 ml of melted nutrient agar which was then poured rapidly onto the surface of a petri plate. After incubation at 37° for 18 hr the numbers of colonies on the plates were counted to determine the 50% inhibitory point for bacterial growth.

To determine the effect of the antibiotic on *in vitro* susceptibility of *E. coli* to antibody and complement, 0.5 ml of a 10^8 concentration of viable bacteria was incubated with an equal volume of a sublethal concentration of cyclacillin, or, as a control, with a similar or 10-fold higher concentration of penicillin G. After an incubation of 30–120 min at 37°, the bacteria were washed several times with saline and used as the target for complement-dependent antibody-mediated bacteriolysis. For this purpose hyperimmune rabbit serum was obtained from animals injected three to four times over a 2-month period with heat-killed *E. coli* (10^8 bacteria per injection). Sera obtained on the 10th day after the last injection were heated for 20 min at 56°. These sera showed a bacteriolytic titer of 1:2048 when added in 0.1 volumes to an equal volume of complement (guinea pig serum diluted 1:20) and 10^8 *E. coli* in 0.1 ml of saline until no killing was evident without complement. In addition, dispersed cell suspensions of the spleens of mice immunized 5 to 6 days ear-

lier by ip injection with heat-killed *E. coli* (10^8 bacteria) were prepared in sterile Hanks' solution by the usual "teasing" technique. These cells were used in a direct bacteriolytic plaque assay for enumerating specific anti-*E. coli* plaque forming cells (PFC) exactly as described elsewhere (5). PFCs appearing in agar plates containing spleen cells from immunized mice and viable *E. coli* were considered due to 19S IgM-secreting immunocytes. No PFCs appeared without complement.

Results. Increased susceptibility of *E. coli* to antibody-complement-mediated bacteriolysis was evident after incubation with 1–2 μ g of cyclacillin for a time as short as 60–120 min. The concentration of cyclacillin was one-fifth to one-tenth the MIC. As can be seen in Table I, use of untreated *E. coli* as the target bacteria for the serologic tests gave a titer of approximately 1:1600 (50% inhibition) when incubated with antiserum and guinea pig complement. Use of the cyclacillin-treated *E. coli* as the target antigen in the serologic bacteriolytic tests gave a titer of 1:12,800 to 1:25,600 when incubated in the same manner with the antiserum and complement. *E. coli* incubated with the same doses of ampicillin also showed markedly increased susceptibility to antibody-mediated lysis; in general, these treated bacteria *in vitro* gave average titers

slightly lower than those obtained with the cyclacillin-treated bacteria. *E. coli* incubated for 60 min with either saline or penicillin G at the same or a 10- to 50-fold higher concentration resulted in a bacterial preparation which gave essentially the same titer as untreated *E. coli*. Increased lysis occurred when the *E. coli* were incubated for longer time periods with the 1- to 2- μ g dose of cyclacillin or ampicillin, but not penicillin G. Maximum lysis occurred after 4–8 hr of incubation at 37°.

E. coli incubated with cyclacillin for 60–240 min at 37° prior to washing and incorporation into agar plates for the PFC assay resulted in many more bacteriolytic plaques. As is evident in Table II, splenocytes from immunized mice showed approximately 300–400 PFCs per million cells tested. When the same numbers of splenocytes were incubated in agar plates containing cyclacillin-treated bacteria, the number of PFCs was usually at least three- to fourfold higher (1500 to 2000 PFCs per million splenocytes). Although many of the bacteriolytic plaques were about the same size as those observed in plates containing untreated *E. coli*, it is important to note that about 30% were relatively smaller (approximately one-half to one-third the size of those observed on plates containing untreated bacteria). However, even if only

TABLE I. EFFECT OF ANTIBIOTICS ON SUSCEPTIBILITY OF *Escherichia coli* TO ANTIBODY-MEDIATED KILLING IN THE PRESENCE OF GUINEA PIG COMPLEMENT.^a

Antibiotic	Concentration (μ g)	Antiserum dilution ^b								
		1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800	1:25,600	0
None		0	0	±	2+	4+	4+	4+	4+	4+
Cyclacillin	1.0	0	0	0	0	0	±	1+	3+	4+
	5.0	0	0	0	0	0	0	0	±	3+
	10.0	0	0	0	0	0	0	0	0	2+
Ampicillin	1.0	0	0	0	±	2+	4+	4+	4+	4+
	5.0	0	0	0	0	±	2+	4+	4+	4+
	10.0	0	0	0	0	0	0	±	3+	3+
Penicillin G	10.0	0	0	±	2+	4+	4+	4+	4+	4+
	50.0	0	0	±	2+	4+	4+	4+	4+	4+

^a Bacteria (10^8) were incubated with indicated dose of antibiotic at 37° for 2 hr, washed, and then incubated with serial dilutions of rabbit anti-*E. coli* antibody plus guinea pig complement diluted 1:20 for 18 hr at 37°; cultures with antibiotic only (no serum or complement) resulted in bacterial growth comparable to that in last column (3+ or 4+).

^b 0 = no growth; ± = trace growth; 2+ or 3+ = slight to moderate growth (20–50% inhibition); 4+ = turbid growth (no inhibition).

TABLE II. INFLUENCE OF ANTIBIOTICS ON SUSCEPTIBILITY OF *Escherichia coli* TO ANTIBODY-MEDIATED LYSIS IN BACTERIOLYTIC PLAQUE ASSAY ON AGAR GEL CONTAINING SPLENOCYTES FROM IMMUNIZED MICE AND COMPLEMENT.

Antibiotic	Number of PFCs per 10 ⁶ spleen cells ^a		
	Large	Small	Total
Controls)	315	58	373
	±31	±8	±46
in (1-2 µg)	1410	455	1865
	±295	±48	±342
lin (1-2 µg)	868	305	1173
	±214	±76	±292
n G (10-20	325	56	381
	±28	±9	±36

^aAverage number of bacteriolytic plaques for five more agar plates, each containing 10⁶ spleen cells from mice immunized 5 days earlier with *E. coli* LPS; small plaque = smaller than 0.05 diameter; agar plates contained inoculum of *E. coli* incubated for 120 min with indicated antibiotic before washing and plating. No PFCs appeared in control plates with bacteria only and no cells.

PFCs were counted, the increase in lysis was still approximately three times more than that observed with untreated *E. coli* incubated with a 10 times dose of penicillin G still resulted in the same number of PFCs as that observed with bacteria incubated in saline only.

Discussion. Earlier studies showed that in ampicillin treatment of *Staphylococcus aureus* results in enhancement of subphagocytosis by normal mouse peritoneal cells (6). This enhanced phagocytosis was not due to a direct synergism between antibiotic with the phagocytic cells.

This model system differed significantly from studies by other investigators in the addition of an antibiotic to an incubation mixture containing a target organism and phagocytes resulted in increased uptake of microorganisms. In such situations the antibiotic apparently served only to inhibit binary division of the bacteria. In the present study, as well as in previous studies in the laboratory with phagocytic cells (6), *E. coli* was incubated with the target organism for a specific length of time and then microorganisms were then washed. It is likely that the exposure of the bacteria to antibiotic *in vitro* at 37° resulted in

subtle changes in bacterial surface structure and/or physiology. Since penicillin G and its derivatives are known to alter and affect mainly cell wall metabolism, resulting in altered cell wall structure, it seems likely that a sublethal dose of an antibiotic such as cyclacillin which shows *in vivo* and *in vitro* efficacy against *E. coli* may result in surface alterations. Thus lesions on the surface of the bacteria may occur which cannot be detected by usual means, including detecting inhibition of cell growth. Nevertheless, these subtle changes may make the organism more susceptible to lysis by antibody and complement and/or reduce the effectiveness of cell surface repair after incubation.

It is important to note that these results are relatively similar to a recent study with mammalian cells by Segerling *et al.* (7). In that system, the ascitic forms of two antigenically distinct guinea pig hepatomas showed marked enhancement of susceptibility to killing by antibody plus guinea pig complement after *in vitro* incubation with small doses of chemotherapeutic agents. The increased killing was dependent on the drug dose and not on increased antigenic expression or fixation of the early components of guinea pig complement. The beneficial effects of various chemotherapeutic agents, including antibiotics, in the treatment of microbial infections might be similar to that proposed for the treatment of hepatomas by drugs, i.e., increased susceptibility to killing by antibody plus complement occurs after prior incubation with small amounts of a chemotherapeutic agent (7). Thus the present study suggests a possible explanation for the earlier results concerning increased susceptibility of antibiotic-treated *E. coli* to host factors and provides a possible mechanism for the greater *in vivo* efficacy of some antibiotics as compared to *in vitro* efficacy. Further studies are in progress concerning the relationship of organism susceptibility to immune factors after treatment with antibiotics.

Summary. Incubation of *Escherichia coli* with sublethal concentrations of the semisynthetic penicillins cyclacillin and ampicillin substantially increased the susceptibility of the bacteria to subsequent lysis by serum

antibody or specifically immunized murine spleen cells in the presence of guinea pig complement. The effect was dependent on the dose of the antibiotic and the length of incubation time and appeared to be due to subtle alterations in the bacterial surface which rendered the organisms more susceptible to immunologic lysis. These results provide a possible mechanism for the greater *in vivo* efficacy of some antibiotics as compared to their *in vitro* activity.

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Effect of Streptozotocin Diabetes on Selected Enzymatic Activities in Rat Urine (39534)

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Patients afflicted with certain pathological conditions, especially those of the kidneys, excrete in their urine significantly greater than normal levels of certain enzymatic activities, among them such acid hydrolases as α -D-glucosaminidase (*N*-acetylglucosaminidase, EC 3.2.1.30), β -D-galactosidase (EC 3.2.1.23), and β -D-glucuronidase (EC 3.2.1.31) (1-4). Experimental and given nephrotoxic agents also produce urine containing acid hydrolase activities at well above control values (5, 6). The urinary levels of acid hydrolase activities are presumably of lysosomal origin, and are thought to be linked to the process of destruction of kidney tissue (2, 3). Recent reports indicate that diabetic patients excrete elevated levels of urinary lysozyme (EC 3.2.1.17), alkaline phosphatase (EC 3.1.3.2), *N*-acetylglucosaminidase, β -galactosidase, and α -D-glucose (EC 3.2.1.20) compared with non-diabetic subjects (2, 7, 8). Further studies are needed to determine the source of the elevated acid hydrolases from diabetic patients and to find out if there is a relationship between the levels of the activities of urinary enzymes and the development of some of the vascular lesions of diabetes (8). In this paper, we suggest that streptozotocin-diabetic rats may provide a model for such studies. We report that these animals excrete significantly elevated levels of urinary *N*-acetylglucosaminidase, acid phosphatase, α -L-fucosidase (EC 3.2.1.51), β -D-galactosidase, and α -D-mannosidase (EC 3.2.1.24) per 24 hr compared to controls. Diabetic rats also excrete significantly elevated levels of the nonlysosomal enzymes alkaline phosphatase (EC 3.1.3.1) and glutamate-oxaloacetate transaminase (GOT, EC 2.6.1.1) per 24 hr than controls. Insulin treatment of the diabetic rats reduced the urinary levels. Address inquiries to this author at the Department of Physical Science.

urinary enzymatic activities to control levels.

Experimental procedures. Chemicals. pNP (*P*-nitrophenyl)- α -D-mannoside, pNP- β -D-galactoside, pNP- α -L-fucoside, and pNP- β -D-2-acetylamino-2-deoxyglucoside were from Sigma Chemical Co. pNP-phosphate and GOT, LDH-L, and LDH-P Fast-Pack reagent kits were from Calbiochem. Protamine-zinc insulin was from Eli Lilly. Streptozotocin was from the Upjohn Co. Other reagents used were of the highest available purity.

Preparation of urine samples. Holtzman male Sprague-Dawley rats (100-300 g) were made diabetic by injection of 60 mg/kg of streptozotocin (in 0.1 *N* citrate, pH 4.5, administered via a tail vein). Three or more days after injection, samples of urine from diabetic and control rats were collected for 24 hr in bottles kept in ice. All animals had access to water. A group of diabetic rats and a control group were fed Purina rat chow during the collection period (fed group); a second group of diabetic rats and their controls were fasted during the urine collection period (fasted group). To induce nondiabetic polyuria, one group of fasted normal rats was given drinking water containing 30 g of glucose and 1.25 g of sodium saccharin/liter (11) and another group of fed normal rats was given drinking water containing 0.5% (w/v) NaCl.

The urine samples were centrifuged for 10 min at 480g and the pellet discarded. Ammonium sulfate (390 mg/ml of urine) was slowly added to the urine and the mixture was stirred for 20 min at 4°, then centrifuged for 30 min at 13,000g. The pellet was suspended in a minimal amount of 0.1 *M* acetate buffer, pH 5, then dialyzed for 4 hr against the same buffer and adjusted to 25 ml total volume with buffer prior to assay.

Assays. Blood sugar levels were estimated by the *o*-toluidine method (12). Data ob-

tained from diabetic rats having blood sugar values less than 300 mg/100 ml of serum were not used. Protein levels were estimated using the biuret method (13).

Assays of acid hydrolase activities were conducted using 0.1 M acetate buffer adjusted to pH 5.0 for α -L-fucosidase, β -D-galactosidase, and *N*-acetylglucosaminidase, pH 4.6 for α -D-mannosidase, and pH 4.2 for acid phosphatase. One milliliter of buffer containing a 2 mM concentration of the appropriate substrate was mixed with 1.0 ml of urine preparation. This mixture and controls (1 ml of urine preparation plus 1 ml of buffer; 1 ml of buffer-substrate plus 1 ml of water) were incubated for 30 min at 37°. One milliliter of 10% trichloroacetic acid was added and the mixture was centrifuged. An equal volume of 0.5 M NaOH was added to the clear supernatant fraction and the absorbance at 410 nm was read. Alkaline phosphatase activity was assayed in 0.5 M glycine buffer, pH 10.5, containing 2 mM pNP-phosphate, incubated as above, then read directly at 410 nm. Calbiochem GOT, LDH-L, and LDH-P Fast-Pack kits were used as directed (14) to assay for GOT and lactate dehydrogenase activities in the urine preparations. In all cases, the activities measured were proportional to the

amount of preparation assayed.

Results and discussion. The activities of acid phosphatase, alkaline phosphatase, *N*-acetylglucosaminidase, and β -D-galactosidase are sufficiently great to permit their detection in crude, dialyzed rat urine. However, the data from assays made using the enzyme activities precipitated from the urine by ammonium sulfate treatment were more precise. Concentration of the urinary protein by ammonium sulfate treatment was prerequisite to the measure of GOT and α -mannosidase activities.

We were unable to detect lactate dehydrogenase activity in the urinary protein fraction. All enzymatic activities were lost after tubes containing the urine preparations were placed in a boiling-water bath for 5 min. Sephadex G-200 column chromatography revealed that most of the urinary acid hydrolase activity was associated with the protein fraction having molecular weights between 100,000 and 220,000. Urines from rats treated with nephrotoxic agents possess glycosidases with similar molecular weights (15).

As seen in Table I, our fed diabetic rats produced almost 12 times more urine than fed controls per 24-hr period, and this urine contained activities of those enzymes of Ta-

TABLE I. ACTIVITIES OF SELECTED ENZYMES IN URINES FROM NORMAL AND FROM STREPTOZOTOCIN-DIABETIC RATS^a

	Normal, fed	Diabetic, fed	Normal, fed, 0.5% saline ^b	Normal, fasted	Diabetic, fasted	Normal, fasted + saccharin ^c
Urine volume (ml/24 hr)	11 ± 2	129 ± 5	22 ± 4	9 ± 2	21 ± 3	190 ± 12
Protein (mg/24 hr)	39 ± 9	290 ± 98	53 ± 7	27 ± 2	34 ± 4	30 ± 4
<i>N</i> -acetylglucosaminidase	2.0 ± 0.2	13.2 ± 1.2	3.1 ± 0.6	1.5 ± 0.3	4.1 ± 0.5	1.6 ± 0.2
<i>N</i> -acetylglucosaminidase, untreated samples ^d	1.1 ± 0.4	8.9 ± 0.9	—	—	—	—
Acid phosphatase	12 ± 2	86 ± 9	23 ± 4	9 ± 2	27 ± 6	9 ± 2
Acid phosphatase, untreated sample ^d	26 ± 4	121 ± 23	—	20 ± 2	40 ± 3	—
Alkaline phosphatase	40 ± 10	160 ± 20	40 ± 10	30 ± 10	60 ± 10	22 ± 10
α -L-Fucosidase	1.2 ± 0.1	3.8 ± 0.3	—	0.8 ± 0.1	2.1 ± 0.2	1.2 ± 0.1
β -D-Galactosidase	4.1 ± 0.4	16.4 ± 2.5	5.4 ± 1.0	2.8 ± 0.4	5.3 ± 0.5	2.4 ± 0.2
β -D-Galactosidase, untreated samples ^d	1.5 ± 0.6	10.8 ± 3.4	—	—	—	—
α -D-Mannosidase	0.9 ± 0.1	4.2 ± 0.6	1.0 ± 0.3	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
Glutamate-oxaloacetate transaminase	0.11 ± 0.02	2.3 ± 0.9	0.17 ± 0.13	0.06 ± 0.02	0.23 ± 0.06	—

^a Average values ± standard errors are presented for selected enzymatic activities in ammonium sulfate-precipitated urinary protein, prepared and assayed as described in the text. *N* = 10 in each case. Lactate dehydrogenase activity was not detected in our samples. Activities are reported as nanomoles of pNP/30 minute in 24-hr samples, except for GOT; activities of GOT are given as nanomoles of oxaloacetate/minute in 24-hr samples.

^b The drinking water of this group contained 0.5% (w/v) sodium chloride.

^c The drinking water of this group contained 30 g of D-glucose and 1.25 g of sodium saccharin/liter.

^d Urines were dialyzed for 4 h against deionized water. For fasted groups, *N* = 24. For fed groups, *N* = 15-19. Urine was collected from pairs of normal control rats.

that were elevated about sixfold over control values. Our fasted diabetic rats produced urinary enzymatic activity values and urine volumes that were about two-fold greater than those yielded by fasted rats and controls in 24 hr ($P_{av} < 0.005$ and $P < 0.001$, respectively). Urines from fasted diabetic rats and from fed diabetic rats both contained significantly less enzymatic activity per milliliter of urine than the control, as may be calculated from the data in Table I. The average specific activities of the enzymes listed in Table I in urine from diabetic rats were twofold greater than that of controls; those of the fed diabetic rat urine were 0.66 that of control. Values for the levels of enzymatic activities in urines collected from rats in the first 3 to 30 days after injection with streptozotocin were similar. The 24-hr urinary volume and the levels of the urinary acid hydrolase activities (and the concentration of urinary glucose) returned to control when fed diabetic rats were treated daily with 2 to 4 units of insulin per cent. Upon cessation of insulin treatment the 24-hr urinary volume and the level of enzymatic activities contained therein initially increased, reaching pretreatment levels about 3 days after the insulin injection was stopped. Results for *N*-acetylglucosaminidase and α -L-fucosidase, which are representative of the behavior of the acid phosphatases, are shown in Fig. 1.

The above data suggest a strong correlation between the volume of urine produced and the levels of urinary enzymatic activity excreted per 24 hr. Indeed, a linear relationship between the volume of urine produced by an individual rat and the level of enzymatic activity contained in that urine was readily apparent when these values for individual rats were graphed. Data from a minimum of 40 animals were plotted for each enzyme studied. Least-squares analysis of the data for each enzyme yielded parameters for a linear equation which were essentially the same as those which may be calculated from the data of Table I. Correlation coefficients (r^2) ranged from 0.73 to 0.88. The parameters for two equations are shown in Fig. 1.

To determine if the increased levels of

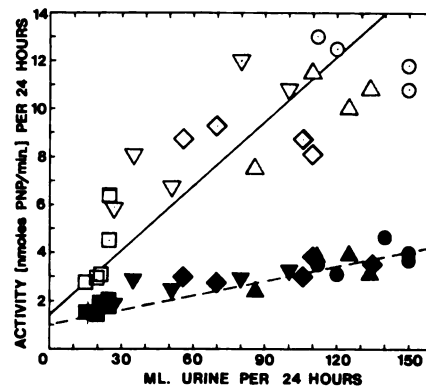


FIG. 1. The effect of insulin upon the excretion of daily total urinary acid hydrolase activity and upon urinary volume produced by fed, streptozotocin-diabetic rats. Ammonium sulfate preparations of urines from five diabetic animals collected at the appropriate times were studied. Mean enzyme activity excreted per 24 hr is plotted against the respective urine volume obtained from each animal for the enzymes *N*-acetylglucosaminidase (open symbols) and α -L-fucosidase (closed symbols). The lines derived from the least-squares equations obtained from analyses of all data (including that from fed and fasted normal rats, fed normal rats given 0.5% saline solution to drink, and fed and fasted diabetic rats) for *N*-acetylglucosaminidase (—) and α -L-fucosidase (---) are also shown. The symbols represent values obtained from fed, streptozotocin-diabetic rat urines: prior to insulin treatment (\circ , \bullet); during insulin treatment (\square , \blacksquare); 1 day after cessation of insulin treatment (∇ , \blacktriangledown); 2 days after insulin treatment (\diamond , \blacklozenge); and 3 days after insulin treatment (\triangle , \blacktriangle). Similar results were obtained with acid phosphatase, β -D-galactosidase, and α -D-mannosidase.

urinary enzymatic activities are related to diabetes or if large amounts of urinary enzymes are yielded by any animals which produce large volumes of urine, we attempted to induce normal rats to produce large quantities of urine by giving them either 0.5% NaCl (w/v) or a glucose and saccharine (11) mixture in their drinking water. Rats given the saline solution produced urinary volumes and enzymatic activities similar to those of fasted diabetic rats. However, rats given glucose and saccharin in their drinking water produced about 17 times more urine in 24 hr than controls, but their urine contained levels of enzymatic activities similar to control values, as seen in Table I. Therefore, it is diabetes itself that is responsible for certain enzymes existing in the 24-hr

urine of diabetic rats at levels well above those of normal control. The excretion rate of lysosomal and of nonlysosomal enzymes is affected by diabetes. The fact that lactate dehydrogenase, an enzyme found at concentrations above control values in urines from subjects having certain renal disorders (14, 16), is not elevated above control values in diabetic rat urine suggests that the effect of diabetes may be specific to certain cell types as the source of the urinary enzymes.

The source of the urinary enzymes under study is still unknown. This and the effect of the excessive loss of those enzymes upon the organ from which they came will require further study.

Summary. The 24-hr urine collected from streptozotocin-diabetic rats contained activities of acid phosphatase, alkaline phosphatase, *N*-acetyl- β -D-glucosaminidase, α -L-fucosidase, β -D-galactosidase, glutamate-oxaloacetate transaminase, and α -D-mannosidase that were significantly greater than control values. Lactate dehydrogenase activity was similar in urines from diabetic and from control animals. Results were similar with rats injected with streptozotocin from 3 to 30 days prior to use. The levels of urinary acid hydrolase activities present in diabetic rats returned to control values upon treatment with insulin and became elevated above control values once again upon cessation of insulin treatment. The levels of enzymatic activity measured in diabetic rat urine, except for LDH, were directly proportional to the volume of urine produced. However, normal animals with induced polyuria did not produce urines with enzymatic activities above control values.

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Possible Determinants of Plasma Renin Activity in Infant Rats (39535)

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mature animals of a variety of species), plasma renin activity (PRA) is low as compared to neonates or during fetal development. In the rat, during the first 3 weeks of life, PRA is about five times as high as in the adult (5, 6). The high PRA probably results in part from a decreased rate of disappearance (6) as well as a possible increased rate of secretion. If, in fact, secretory rates are different from what one could expect, an explanation would fit into one of several general categories of possibilities: (a) a stimulus may be present disproportionately in the young to cause an increased rate of secretion; (b) a stimulus may be present which normally suppresses renin secretion; or (c) the kidney is temporarily unresponsive to stimulus inputs and the newborn represents a basal uncontrolled secretory rate. To test these possibilities, it was decided to analyze the responsiveness of the renin-angiotensin system to a series of physical states known to alter renin secretion. More specifically, we have studied the effect of increased salt intake, β -adrenergic blockade, antidiuretic hormone, and a competitive inhibitor of angiotensin (1-sarcosine-3-alanine-angiotensin II, hereafter referred to as P-113) on PRA in infant rats.

Materials and methods. Fourteen- to six-day-old infant rats (14 litters, body weight 22–28 g; mothers, 300 g body weight) were usually used in these studies. This age was chosen because (i) it is well within the 3- to 4-week "critical period"

PRA decreases to mature levels (5, 6); (ii) such animals are large enough to allow for relatively easy venipuncture by puncture of the jugular vein; (iii) about 0.7 ml of blood can be collected, usually by drawing blood from the cut lower vena cava and

abdominal aorta. In one series of studies, however, animals were investigated which ranged in age from birth to 14 days. All animals used in these studies were of the Charles River strain, obtained from a commercial supplier (Arie Lebenstein Laboratory Animals, Yokneam, Israel), or were born in our laboratory to pregnant dams obtained from the same supplier.

When necessary, animals were anesthetized with Nembutal by first administering two-thirds the normal adult dose intraperitoneally. If this amount of anesthetic was not adequate, supplementary small increments were administered. After an experimental maneuver (see below), a laparotomy was performed and the intestinal contents displaced so as to expose the inferior vena cava and aorta. The intestinal wall was quickly wiped dry and the major vessels were cut. The free-flowing blood was then collected in cold EDTA-containing syringes in order to inhibit angiotensinase activity. After transfer to cold tubes and centrifugation, plasma was stored at -20° until analysis of PRA. Renin activity was determined by radioimmunoassay using a micromodification of the New England Nuclear kit. The procedure was in principle the same, but only 50 to 100 μ l of plasma were incubated for 3 hr. Appropriate dilutional adjustments were made so that final concentrations of Ang I were appropriate for sensitive assay. The intra-assay coefficient of variation was found to be 7%, and inter-assay was 11%.

Significance of differences between means of treated and untreated animals was evaluated using a two-tailed *t* test. It was inferred that a difference existed when the likelihood of two means being the same was less than 0.5.

Control animals for most studies were untreated 14- to 16-day-old rats. There were several different experimental groups.

Group 1. Dams were obtained as soon as pregnancy was certain. This time was be-

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tween 2 and 3 weeks before delivery. The animals were given 1% saline exclusively for drinking water. At higher concentrations of salts, 2%, early abortion was observed or the infants died within 1 day of birth. On the 1% saline, however, about 50% or more of the pups survived. The survivors were sacrificed for blood collection at varying time intervals after birth.

Group 2. In this group, the effect of intraperitoneal salt administration on PRA was studied. Fourteen-day-old animals were injected with normal saline for 3 consecutive days at a dose of 10% body weight at each injection. One day later, blood was collected and examined for PRA.

Groups 3 and 4. The ability to suppress renin release by β -blockade was determined in these groups. The pups were injected subcutaneously with propranolol at a dose of 10 mg/kg. The propranolol solution (ABIC, Israel) had a concentration of 1 mg/ml. In group 3, blood samples were taken 2 hr after the injection, while in group 4, blood was collected 3 hr later. Controls for these groups consisted of pups injected with saline alone.

Groups 5 and 6. In these groups, the ability of antidiuretic hormone to suppress plasma renin activity was investigated. Since in the rat it had been suggested that only high doses of ADH lead to suppression of renin (8), two doses of Pitressin in oil (Parke-Davis) were used in these studies. In group 5, each animal received 200 mU, while in group 6, the dose was 2 mU/animal. The drug was administered im with controls being injected with oil alone.

Group 7. In these animals, the competitive inhibitor of angiotensin II, P-113, was administered via a jugular vein. The drug was given as an acute injection in a volume of 0.1 ml of saline containing a total of 20 mg of P-113. Fifteen minutes later, blood was sampled for PRA measurement.

Results. Plasma renin activity of group 1 animals (mothers on a high salt diet) at varying ages after birth are shown in Fig. 1. Using this method of attempting to increase body sodium of infants resulted in no decrease in circulating PRA during the first 14 days of life. In control animals at these ages, control PRA has been found to be in the

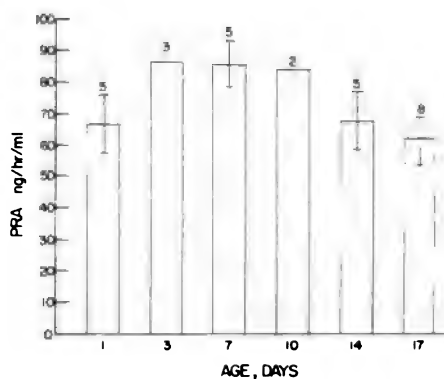


FIG. 1. Plasma renin activity (PRA) as a function of age in animals nursed by mothers receiving 1% NaCl for drinking water. Bars indicate standard error of the measurements, and numbers above the bars indicate the number of determinations at each age.

range of 50–80 ng/hr/ml of plasma and is constant with age. It is not possible to be certain, however, that the infants did in fact have a higher intake of sodium in these studies (10, see Discussion). As a result, we approached the problem in an alternate way. Pups were treated with saline as described in Methods, group 2. Results of these studies are shown in Table I. Saline administration resulted in no change in PRA. The value of 62.1 ± 6.3 ng/hr/ml is not significantly different from the control value.

β -Adrenergic blockade (groups 3 and 4) was induced by subcutaneous injection of propranolol. It has been shown in adults that this method of administration results in a maintained blood concentration, the plateau being reached in less than 30 min (9). In 2-week-old rats, no change in PRA of propranolol-treated animals was found at 2 hr after injection as compared to control animals injected with saline or to uninjected controls (Table I). Since, however, there may be a delay of absorption in infants (11), these studies were repeated with blood being sampled 3 hr after injection. In these animals, it was found that β -blockade also had no significant effect on PRA at this time.

In contrast to treatment by salt or by propranolol, injection of 0.2 unit of ADH in oil resulted in suppression of PRA. The dose was calculated from the data of Gut-

TABLE 1. EFFECT OF VARIOUS AGENTS ON SUPPRESSING PRA IN 2-WEEK-OLD RATS.

Treatment	n	PRA	P
Controls	7	76.1 \pm 7.8	—
	8	62.1 \pm 6.3	N.S.
Propranolol, 2 h	8	40.7 \pm 5.0	—
Propranolol, 3 h	7	50.5 \pm 12.1	N.S.
	18	45.6 \pm 4.2	N.S.
0.002 U	6	73.8 \pm 8.2	—
0.2 U	8	77.7 \pm 14.5	N.S.
	8	38.9 \pm 6.7	<0.01
	5	47.1 \pm 3.4	—
	6	27.8 \pm 39.5	<0.001

and Benzakein (8) who suggested that as a very high dose at "pharmacological levels." A repeat of this study with administration of Pitressin at 1/100 of the original dose level did not suppress PRA.

The final treatment was with P-113. It is that administration of the antagonist to enzyme II results in a marked increase in circulating PRA.

Discussion. The role of various regulatory factors in altering renin secretion of mature animals is shown in Fig. 2. The question arises as to which of these may be physiologically significant in the infant. Accordingly, following criteria would seem to be reasonable prerequisites to assigning a role for a regulatory agent as acting in a significant manner during the neonatal period. (i) Alteration of the quantity of the agent should be predictable changes in PRA. (ii) During the postnatal period, changes should be in the concentration or effectiveness of the agent. (iii) The time of change of the agent should coincide with the time of change in circulating PRA. (iv) The changes in the regulator should be in the appropriate direction to account for the change in PRA. In the neonate, plasma sodium concentration (P_{Na}) is low, and in rats it shows a gradual increase in the postnatal period (Solomon and Bengel, in preparation). At 3 to 4 weeks of age, P_{Na} is still low relative to adult levels. Since this is the "critical period" for reduction of PRA to mature levels, it did not seem that the low P_{Na} by itself is sufficient for the high PRA of the neonate. Since P_K is high in rat pups (12, Solo-

mon and Bengel, in preparation), the action of this ion would be to decrease PRA. Potassium changes in the wrong direction to be a regulator of renin.

Our own studies served further to eliminate sodium as a trigger for increased renin secretion. When the mothers were on a high salt diet, there was no change in PRA of the young (Fig. 1). Comparable results were obtained with fetal lambs, although salt restriction of the mother increased fetal PRA (4). In addition, fetal lambs respond to diuretics (5). It may be argued that the high salt diet has inadequate effect on fetal sodium or on milk sodium, since Dlouha and co-workers found only a small increase in rat milk sodium in mothers fed a high Na diet starting at birth (10). In the mature animal, it is well known that salt loading suppresses PRA. To insure that salt intake was in fact increased, we administered isotonic saline by the intraperitoneal route. PRA was found to be the same as in controls. It does not appear, therefore, that increasing body sodium in the neonate is able to suppress PRA. In newborn dogs, however, peritoneal dialysis produces a significant increase in PRA. As a result, the possibility must be considered that offsetting effects are produced in this experiment, a reduction in PRA by the salt and an increase because of the route of administration.

The results with propranolol are similar in being ineffective in suppressing PRA. One may argue that we did not utilize a high enough dose, but at this level we have always observed bradycardia (unpublished). In addition, we have found that propranolol prevents or reduces the rise in PRA secondary to a stimulus in mature animals (13). In agreement with Dornier *et al.*, we found little suppression of basal PRA in mature animals (14). It has been shown that plasma

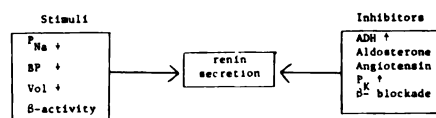


FIG. 2. Diagrammatic representation of some known factors affecting renin secretion. Arrows indicate direction of change of factor.

propranolol reaches a steady-state level 10 min after subcutaneous injection. If one assumes that propranolol would be effective 30 min after injection, one can estimate that if β -blockade reduced renin secretion, one would expect some reduction at 2 hr after injection, since the half-time of destruction is about 26 min in neonates with high PRA (7). At this time, no significant reduction in PRA was found. Since it is known, however, that absorption in the neonate from several sites can be slower than in the mature animals (11), we repeated these studies with blood collections made 3 hr after injection. Again, no significant depression of PRA was observed. As a result, one can suggest that the high PRA of the neonate is not a result of high levels of autonomic stimulation.

In contrast to the failure of salt or β -blockade to affect PRA, 0.2 unit of ADH suppressed renin levels 4 hr after injection. Although this dose has been labeled "pharmacological," to us such characterization of the dose is not appropriate at this time. The hormone was injected in oil and intramuscularly, and one does not know either the rate of absorption or the blood levels attained in our studies as well as in those by others (8). It is of interest that in the dog, "physiological" doses of ADH can suppress renin (15) when given intravenously. It is, however, possible that species differences exist, and, if the effective dose is in fact excessive, this result may have little bearing on a possible role of ADH in suppressing PRA.

With respect to the possibility that ADH is involved in the regulation of PRA, it should be pointed out that it meets criteria presented at the beginning of this discussion. (i) The change in PRA is in the direction predicted for its playing a regulatory role. (ii) Rats are unable to produce a concentrated urine in response to ADH until the third week of life. Thus, the time of ADH effectiveness coincides with the time of onset of mature levels of PRA. Although part of the change in neonatal responsiveness to ADH is a result of anatomical maturation of the thin loops of Henle (16), part of the failure to produce a concentrated urine is the result of a lack of ADH (17). (iii) The changes in the hormone increase during neonatal development are accordingly in the

appropriate direction to assign ADH a regulatory role. In part, then, the high PRA possibly be related to the failure of ADH to suppress renin secretion.

It is not possible, however, to say that ADH ineffectiveness and reduced rate of destruction of renin are the only factors responsible for the high PRA of neonates. It is known that the angiotensin II antagonist P-113, does not affect basal secretion in adults. If, however, a stimulus to renin secretion exists, it has been found that the inhibitor interferes with biological feedback and renin activity increases. Such an effect has been observed with varied stimuli after adrenalectomy (18), vena caval constriction (19), reduction of hypertension of renin (20) and following renal ischemia and subsequent production of acute renal failure (13). In the infant, P-113 administration also markedly increases the level of renin. One possible source of the stimulus in neonatal animals is anesthesia (21). It has been found, however, in control animals in our studies that P-113 alone does not raise renin in anesthetized mature rats (21). In addition, in mature animals we have never observed PRA levels as high as in infants with comparable anesthesia (11). It would, therefore, seem that a stimulus exists to cause secretion of renin but that the nature of the stimulus has not been elaborated by our studies.

Summary. The effect of various experimental maneuvers in altering PRA of neonatal rats was studied. In 2-week-old animals, suppression of PRA is found after salt loading or β -blockade. Infants nursed by mothers receiving 1% saline for drinking water show no changes in PRA from birth to 2 weeks. Intramuscular administration of ADH (0.2 U) in 2-week-old animals suppressed PRA, while P-113 resulted in a marked increase in PRA. These latter results suggest that some stimulus is causing inappropriate secretion of renin, and, in addition, there was some failure of feedback suppression by ADH.

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Heterogeneity of Renin Substrate in Human Plasma: Effect of Pregnancy and Oral Contraceptives (39536)

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There have been few studies of the electrophoretic mobility of renin substrate in plasma and only two¹ reports of the behavior of renin substrate in human plasma (1, 2). Both of these reports indicate that there may be, in human plasma, a small quantity of a second renin substrate with a lesser mobility than the obvious major component, which migrates to a position just behind albumin. Since our last publication, we have modified our technique, making it more effective in showing the presence of small amounts of renin substrate in plasma. With this modified technique, we have found at least five electrophoretically dissimilar renin substrates to be present in human plasma. In addition, we have found significant variations of the pattern of renin substrates in the blood plasma of pregnant women and of women taking oral contraceptive medication.

Methods. Details of our technique are described in our previous publication (2). In brief, the procedure consists of electrophoresis of plasma proteins on a polyacrylamide-gel cylinder, slicing the gel, incubating each slice separately with human renin, EDTA, and BAL in a small volume of saline, and transferring an aliquot of this mixture to a test tube containing the necessary components for radioimmunoassay of angiotensin I. Three modifications of technique have proven to be effective in increasing the "visibility" of small amounts of renin substrate present in blood plasma: (i) increasing the sample size, (ii) prolonging the time of incubation with renin, and (iii) improving the purity of the renin used. We now apply 40 μ l of a 50:50 mixture of plasma and bromphenol blue dye plus sucrose solution (equivalent to 20 μ l of plasma) to the top of each gel prior to electrophoresis, which is

eight times as much plasma as we previously used. In order to do this without overloading the gel and without greatly increasing the depth of the layer applied initially to the top of the gel, we use a gel tube twice the diameter, 10-mm i.d. instead of 5-mm i.d., giving four times the cross-sectional area to which the sample is applied. In a few experiments we have used as much as 100 μ l of sample applied to the gel.

In connection with the second and third modifications of technique listed, if one uses a prolonged incubation with renin (for example 24 hr instead of 2 hr), it is necessary to use a renin preparation which is reasonably free of angiotensinase. Otherwise, during the prolonged incubation, destruction of angiotensin keeps pace with its rate of formation. We have used three different human renin preparations in our experiments, two prepared in our own laboratory and one kindly supplied by Drs. E. Haas and H. Goldblatt. All three were made up as a stock solution in saline equivalent to 1 Goldblatt until ml, which was kept frozen. The final concentration was varied but usually was approximately 0.02 GU/ml of incubation mixture. All three renins were prepared by the technique described by Haas *et al.* in 1966 (3) in their procedure A. Our less pure renin was carried through steps 1-3 of this procedure, which still leaves considerable contamination with angiotensinase. Our purer renin and the one from Dr. Goldblatt's laboratory were carried through steps 1-5 of the Haas procedure, which gets rid of most, but not all, of the angiotensinase present in the kidney extract.

Results. In normal plasma from male or female subjects, there is a single large peak of renin substrate with a mobility slightly less than that of albumin. There is, in addition, a series of very small peaks of renin substrate with lesser mobilities than the major peak. Usually four small peaks appear

¹ Four, if one counts preliminary abstracts by the same authors.

before" the major peak and we have labeled these A, B, C, and D and called the major peak E. Their electrophoretic mobilities relative to the front of the albumin band (R_f alb) are A, 0.05–0.10; B, 0.25–0.30; C, 0.40–0.45; D, 0.50–0.55; and E, 0.75–0.80. Figure 1 shows an example of this pattern in a normal male and also shows how the small more slowly moving peaks (especially B) are made to stand out by increasing the sample size and prolonging the time of incubation with renin. Figure 2 shows the same for the pattern of renin substrates in the plasma of a normal female. In this particular example, peaks C and D are absent, but other female plasmas do show one or both of these peaks. Of the four minor peaks, the second (B) is usually present in the largest quantity and is the one previously reported to be present. It is not yet certain from our experiments whether there are consistently demonstrable minor peaks of renin substrate with electropho-

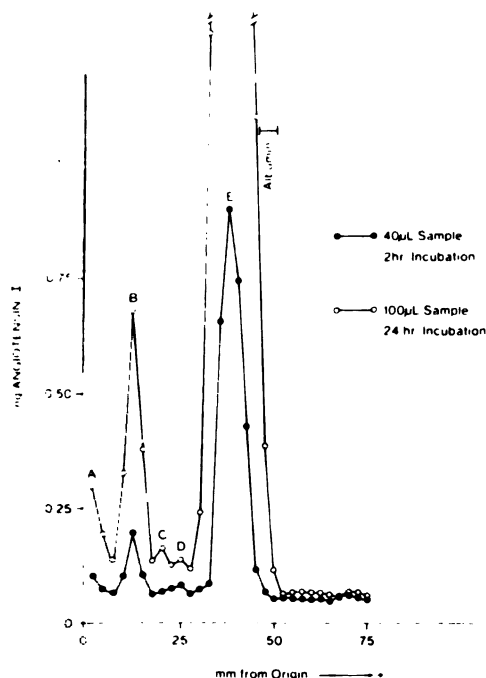


FIG. 1. Pattern of renin substrate after electrophoresis on polyacrylamide gel. Normal male human plasma. The small, slower-moving peaks of renin substrate, especially B, are more prominent when a larger sample and a longer incubation time are used. The position of albumin is indicated by the horizontal bar.

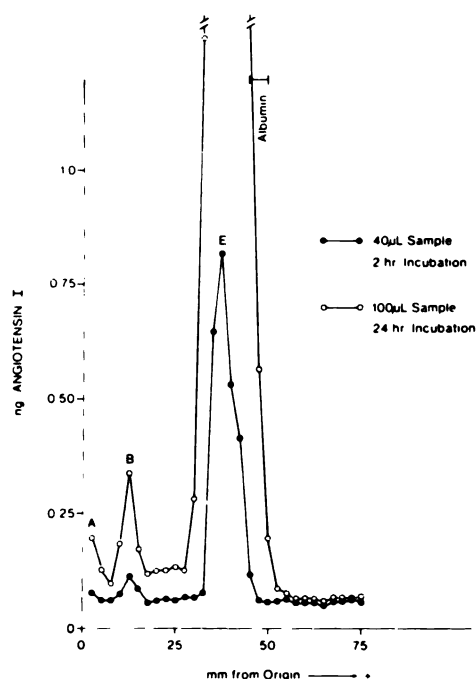


FIG. 2. Pattern of renin substrate after electrophoresis of normal female plasma. Minor peaks show a similar but not identical pattern compared to that of normal male.

retic mobilities greater than that of the major renin substrate peak. In some experiments two or three such rapidly migrating substrates do appear and, if we can confirm their existence, they will be labeled F, G, and H, making a total of eight electrophoretically different renin substrates present in normal human plasma. We have examined the pattern of renin substrates in the plasma of six normal males and eight normal females and found no obvious differences between the individual plasmas.

Effect of oral contraceptives. It has been shown by several investigators (4–10) that the concentration of renin substrate in the blood plasma of women taking oral contraceptive medication (estrogens plus gestagens) is greatly increased. Increases of three to five times normal control values have been reported. We subjected plasma from six women using such medication to the electrophoretic analysis of renin substrate described above and found a distinctly different pattern of renin substrates from that shown by women not taking such medica-

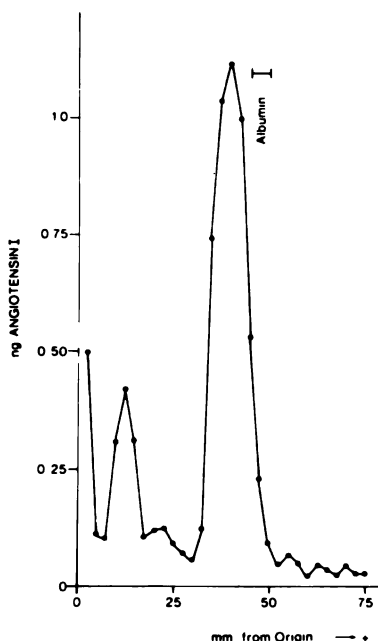


FIG. 3. Pattern of renin substrates in plasma of a woman taking oral contraceptive medication. Note the striking increase of the two slowest-migrating peaks.

tion. An example is shown in Fig. 3. Peaks A and B are especially increased. The major peak (E) may also be increased, but this is not always evident. The pattern of substrates in women taking oral contraceptives is quite characteristic and, although minor variations occur, it can be readily recognized.

Effect of pregnancy. The fact that the total amount of renin substrate increases significantly in pregnant women has been well established (5, 11-16). We subjected the plasma of seven pregnant women to electrophoresis and measurement of renin substrate and found a pattern of renin substrates strikingly different from that of non-pregnant women and also, to a lesser degree, different from the pattern of women taking oral contraceptives. Not only are peaks B, C, and D significantly increased but the bases of all the slow-moving peaks run together so that there is not the usual return to baseline between peaks. An example of the pattern of renin substrates in the plasma of a pregnant woman is shown in Fig. 4. In addition, in the same figure, the effect of omitting the renin during the incu-

bation step is shown. The absence of the peaks when renin is omitted indicates that they are, indeed, renin substrates. All but one of the plasmas from pregnant women which we tested were from pregnant women at term or nearly at term. The only plasma from an earlier stage of pregnancy was from a woman 7 months pregnant and in her case the pattern of renin substrates is closer to that seen in women using oral contraceptives. In general, there seems to be a greater variation in the pattern of renin substrates in pregnant women than in the other groups.

Effect of nephrectomy and of cirrhosis. In addition to the categories described above, we have examined the renin substrate pattern in two other situations in which renin substrate might be expected to be altered: in patients with cirrhosis of the liver and ascites and in patients subjected to bilateral nephrectomy. In two of four cirrhotic patients, the amount of major substrate peak

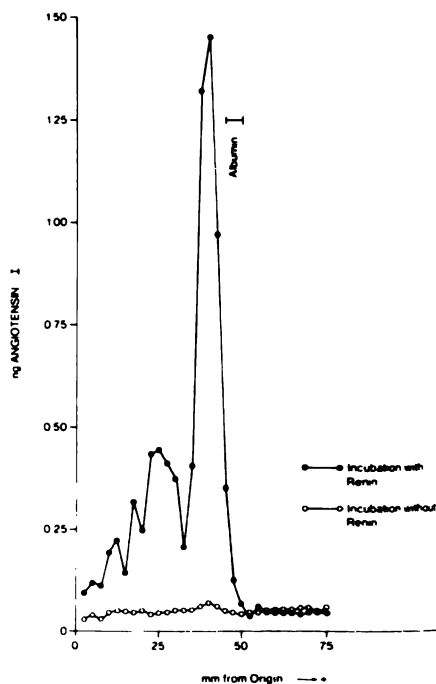


FIG. 4. Pattern of renin substrates in plasma of a pregnant woman. All slow-moving peaks are increased, especially the one migrating behind the major renin substrate peak. Open circles show the absence of angiotensin produced from renin substrate when renin is omitted from the incubation.

was greatly diminished and the minor peaks were also reduced. In three bilaterally resectomized patients, contrary to our expectation, there was not a significant increase in the major peak and the pattern of peaks was not different from normal. *Control experiments.* In order to confirm the presumption that the peaks of renin substrate activity which we observed were in isomeric forms of renin substrate, we had it was necessary to show (i) that they did not appear (or were greatly diminished) if the step involving incubation with renin was omitted, (ii) that the product formed by incubation with renin had the characteristic properties of angiotensin, and (iii) that the peaks could be eliminated (i.e., converted to angiotensin) by prolonged incubation with renin prior to electrophoresis. Figure 4 shows that omission of the renin incubation step does prevent the appearance of the renin substrate peaks. The fact that in all experiments we used antibodies against angiotensin I to detect the end product of the renin reaction (by radioimmunoassay) is itself strong evidence that the material we measured is some form of renin substrate. We found that the product formed (a) is absorbed onto charcoal and (b) is not destroyed by 10-min heating to 95–100°; both properties confirm that the active product is not a protein. In addition, we found that we could eliminate the peaks of renin substrate by a 2-hr incubation of the plasma with human renin, thus converting the substrates to angiotensin prior to electrophoresis of the plasma. In the results of all these tests support the presumption that the peaks of activity we observed in fact, variants of renin substrate. *Discussion.* Our results show that there are five or more electrophoretically distinguishable renin substrates in human plasma. In 1963, Skeggs *et al.* (17) reported the isolation of five or more forms of renin substrate from hog plasma by means of DE-cellulose chromatography. However, the method used by Skeggs *et al.* involved preliminary chemical treatment including exposure of the plasma to a pH of 12 and it has been pointed out that this treatment may have altered the substrate molecule, so that it is uncertain to what

extent the results correspond to the situation in unaltered plasma (18). It should be emphasized that in our work the plasma is not exposed to extreme conditions prior to or during electrophoresis on polyacrylamide gels. The buffer we used for electrophoresis is Tris-glycine buffer, pH 7.9.

Whether the heterogeneous nature of renin substrate in human plasma has any biological significance is unknown. It is most interesting that situations such as pregnancy and medication with oral contraceptives result in alterations of the relative amounts of the different renin substrates present. Skinner *et al.* (19) reported that renin substrate in plasma from pregnant and nonpregnant women behaved identically when chromatographed on columns of DEAE-Sephadex. The difference between their results and ours can probably be explained by the different methods used for separation of plasma proteins. If some biochemical differences between the variants of renin substrate can be shown, e.g., differences in rate of reaction with renin, this could be significant, since it might affect the quantity of angiotensin generated by circulating renin *in vivo*. In any case, now that the existence of variants of renin substrate has been shown, future investigations of possible clinical correlation will have to take account of qualitative as well as quantitative variations of this substance.

Summary. The electrophoretic mobility of renin substrate in human plasma was determined by electrophoresis of the plasma on a cylinder of polyacrylamide gel, followed by slicing the gel, incubation of each slice with human renin, EDTA, and BAL, and determination of the angiotensin formed by radioimmunoassay. In the plasma of normal males and females, a single large peak of renin substrate was found with an electrophoretic mobility somewhat less than that of albumin. In addition, a series of three or four very small peaks of renin substrate with lesser electrophoretic mobility were also observed, the second peak from the origin being the largest of the minor peaks. The peaks, in order of increasing mobility were labeled A through E, E being the large major peak of renin substrate. Occasionally, but not consistently,

observed were two or three small peaks of renin substrate with electrophoretic mobilities greater than that of the major peak. Thus, there are five and possibly as many as eight electrophoretically distinguishable renin substrates present in normal human plasma.

In women taking oral contraceptives the pattern of renin substrates is different from that of women not taking such medication. Peaks A and B are significantly increased. In pregnant women a different pattern of renin substrates is found; the minor peaks being markedly increased, especially B, C, and D. Plasma of patients with cirrhosis of the liver and of patients subjected to bilateral nephrectomy were also examined. The pattern of renin substrates in these did not differ significantly from normal, except that the quantities of all the variants of renin substrate, including the major peak, were greatly reduced in some of the patients with cirrhosis.

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Effect of Thyroid Hormone Excess and Deficiency on Serum Thyrotropin in Rats Immunized Passively with Antiserum to Somatostatin¹ (39537)

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It is now known that somatostatin, in addition to inhibiting the release of growth hormone (GH) (1-5), also inhibits the secretion of several other hormones, among them thyrotropin (TSH), insulin, glucagon, and epinephrine, in various animal species, both *in vivo* and *in vitro* (6-12). However, the physiological role of somatostatin in regulating the secretion of these hormones is still unclear.

Previously, a specific antiserum to somatostatin was generated in sheep in our laboratory (13). This antiserum has been used to study the effect of endogenous somatostatin on the secretion of GH and TSH. In order to clarify the possible physiological role of that hormone in the regulation of GH and TSH secretion, it has been found that passive immunization with the antiserum to somatostatin prevented a stress-induced decrease in serum GH (13), augmented the release of GH after exposure to cold (14), increased the TSH response to thyrotropin-releasing hormone (TRH) (15). These findings suggested that somatostatin plays a physiologically important role in the regulatory mechanism of the secretion of TSH.

To examine the interaction between somatostatin, TRH, and thyroid hormones in the regulation of TSH secretion we studied the effect of triiodothyronine (T₃) on the TRH-stimulated TSH concentration in rats passively immunized with anti-somatostatin (anti-SS).

Materials and methods. Male Charles River CD-1 strain rats were used throughout the experiments. They were maintained in

animal quarters with controlled temperature (24°) and illumination (0500-1900 hr) and were given free access to tap water and Purina laboratory chow.

Experiment 1. Rats weighing 300-360 g were divided into three groups of 16 rats each. The first group was injected with 0.1 ml of 0.9% saline, the second with 100 ng of T₃, and the third with 200 ng of T₃/100 g BW sc. These three groups were divided into two subgroups of eight animals each. Each subgroup was then injected *iv* under ether anesthesia with either 2 ml of anti-SS or 2 ml of normal sheep serum (NSS) at the same time as T₃ was given. Two hours later all animals were injected *iv* with 200 ng of TRH/rat. Blood was drawn from the jugular vein before and 5 min after TRH.

Experiment 2. Rats weighing 300-350 g were divided into three groups of 10 rats each. The first group was injected with 0.1 ml of 0.9% saline, the second with 1 µg of T₃, and the third with 10 µg of T₃/100 g BW sc. These three groups were divided into two subgroups of five animals each. These subgroups were then given *iv* either 2 ml of anti-SS or 2 ml of NSS at the same time as T₃. Two hours later all rats were injected *iv* with 200 ng of TRH/rat. Blood was drawn from the jugular vein before and 5 min after TRH.

Experiment 3. Thirty immature rats weighing 70-80 g were thyroidectomized under Surital anesthesia (3.5 mg/100 g BW) and then divided into three groups of 10 animals each. These groups were then divided into two subgroups of five animals each. These subgroups were injected *iv* with 1 ml of anti-SS or 1 ml of NSS 1, 3, or 7 days after the thyroidectomy. Two hours later the animals were killed by decapitation, and blood was collected from the trunk.

Another 30 rats were sham-operated and divided into groups in the same manner as

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the thyroidectomized animals. Each subgroup was injected iv with either 1 ml of anti-SS or 1 ml of NSS 1, 3, or 7 days after the operation. Two hours after the injection, the blood was collected. In addition, 10 intact animals were divided into two groups, one of which was injected with anti-SS and the other with NSS, and blood was collected 2 hr after injection. TSH was determined by radioimmunoassay (RIA) as described by Kiefer *et al.* (16), with the use of NIAMDD rat pituitary kit for rat TSH.

The antiserum to somatostatin was generated in sheep as described elsewhere (17). The antiserum used in the present study (No. 774) bound 85% of ¹²⁵I-labeled-Tyr¹-somatostatin (sp act, 330 μ Ci/ μ g) at 1:70 dilution. At 1:14,000 dilution of antiserum, the tracer-antibody binding was inhibited by unlabeled cyclic somatostatin in a dose-related manner in a range from 32 to 4096 pg/tube. There were no cross-reactions with TRH, luteinizing hormone-releasing hormone (LHRH), rat luteinizing hormone (LH), follicle-stimulating hormone (FSH), TSH, GH, or prolactin. It was shown earlier that the administration of this antiserum did not interfere with the determination of rat TSH (15).

The means of serum TSH levels for each group were compared by using the Student's *t* test or Duncan's new multiple range test (18).

Results. Experiment 1 (Fig. 1). In the first group of rats, injected with saline and not with T₃, the basal serum TSH level was significantly higher in the animals pretreated with anti-SS than in those given NSS (1.25 ± 0.23 vs 0.54 ± 0.05 μ g/ml, $P < 0.01$). There was a significant increase in serum TSH 5 min after TRH in both anti-SS- and NSS-treated animals, and the mean TSH level after TRH was significantly higher in the anti-SS-treated animals than in those pretreated with NSS (3.14 ± 0.23 vs 2.02 ± 0.21 μ g/ml, $P < 0.01$).

In the second group injected with 100 ng of T₃/100 g BW both the basal and post-TRH serum TSH levels were suppressed to about half of those in respective control animals. The basal as well as the TRH-stimulated TSH levels were significantly higher in the anti-SS-treated rats than in the NSS-treated rats (0.50 ± 0.07 vs 0.22 ± 0.04 μ g/ml, $P < 0.01$, and 1.73 ± 0.15 vs 0.91 ± 0.04 μ g/ml, $P < 0.01$, respectively).

In the third group of rats treated with 200 ng of T₃/100 g BW the basal serum TSH

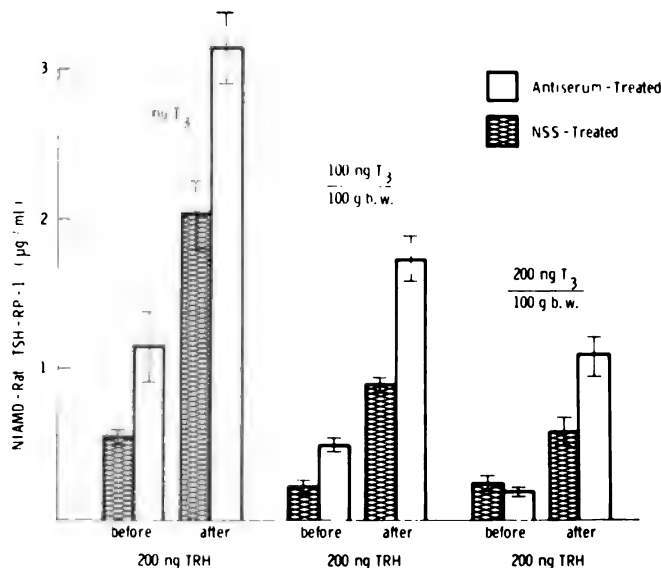


Fig. 1. Effect of T₃ on serum TSH levels before and 5 min after TRH in rats pretreated with sheep antiserum to somatostatin or normal sheep serum (NSS). Both T₃ and serum were administered 2 hr prior to the injection of TRH. The significance of differences between the corresponding TSH values in the antiserum- and NSS-treated groups was calculated using Student's *t* test (** $P < 0.01$).

did not differ between the subgroup pretreated with antiserum and that pretreated with NSS (0.19 ± 0.02 vs 0.24 ± 0.02 $\mu\text{g/ml}$). In both subgroups, the TSH level increased significantly after TRH. The intraoperative serum TSH level in response to TRH was significantly greater in anti-SS-pretreated rats (1.09 ± 0.13 vs 0.10 $\mu\text{g/ml}$, $P < 0.05$).

Experiment 2. As in the previous experiment, the first group of animals which received T₃, both the basal and the stimulated TSH levels were significantly higher after pretreatment with anti-SS than with NSS (1.00 ± 0.11 vs 0.58 ± 0.05 $\mu\text{g/ml}$, $P < 0.01$, and 3.97 ± 0.31 vs 0.41 $\mu\text{g/ml}$, $P < 0.01$, respectively). Administration of 1 μg of T₃/100 g BW totally suppressed the basal serum TSH and blocked the TSH response to TRH; there was no difference in either the basal or the post-TRH TSH levels between rats pretreated with anti-SS or NSS (0.25 ± 0.02 vs 0.25 ± 0.02 $\mu\text{g/ml}$ and 0.28 ± 0.03 vs 0.27 ± 0.03 $\mu\text{g/ml}$). Similar results were obtained in the animals treated with 10 μg /100 g BW (0.22 ± 0.02 vs 0.22 ± 0.02 $\mu\text{g/ml}$ and 0.27 ± 0.03 vs 0.25 ± 0.04 $\mu\text{g/ml}$).

Experiment 3 (Fig. 2). In the unoperated control rats, the basal serum TSH concentration was significantly higher after pretreatment with anti-SS than with NSS (1.15 ± 0.19 vs 0.05 $\mu\text{g/ml}$, $P < 0.01$). There was a marked rise in serum TSH on the day of thyroidectomy, with a further increase on the following days after the operation. The TSH level was significantly higher in the anti-SS-pretreated animals both on Day 1 (1.15 ± 0.19 vs 0.05 $\mu\text{g/ml}$, $P < 0.01$) and on Day 3 (2.32 ± 0.07 vs 1.01 ± 0.07 $\mu\text{g/ml}$, $P < 0.01$, and 3.33 ± 0.70 vs 0.88 ± 0.08 $\mu\text{g/ml}$, $P < 0.01$, respectively). However, on Day 7 after thyroidectomy there was no difference in TSH between the anti-SS- and NSS-treated groups (6.99 ± 0.36 vs 6.88 ± 0.36 $\mu\text{g/ml}$).

In sham-operated rats the TSH levels were significantly lower on Days 1, 3, and 7 after operation than in the corresponding group of thyroidectomized animals treated with anti-SS and NSS, respectively. There was no difference in the corresponding anti-

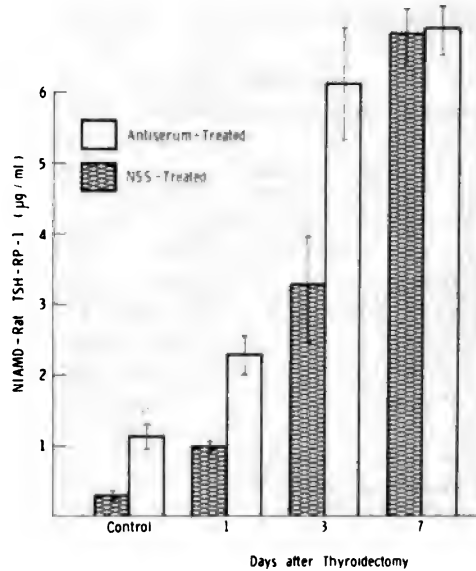


FIG. 2. Serum TSH levels in rats pretreated with antiserum to somatostatin or normal sheep serum (NSS) 1, 3, or 7 days after thyroidectomy and in non-operated control rats. The significance of differences between the corresponding TSH values in the antiserum- and NSS-treated groups was calculated using Student's *t* test (* $P < 0.05$, ** $P < 0.01$).

SS- and NSS-treated groups between non-operated and sham-operated animals, and the serum TSH levels were significantly higher in the anti-SS- than in the NSS-treated rats on Days 1, 3, and 7 after the operation (results not shown). Thus, the stress of the operation did not cause any nonspecific differences between the groups.

Discussion. The present study confirms and extends our previous observation that passive immunization by specific antiserum to somatostatin increases the basal as well as the TRH-stimulated TSH secretion in rats (15). This phenomenon is most probably due to the nullification of the action of endogenous somatostatin by passive immunization.

TRH stimulates TSH secretion presumably by binding to specific receptor sites on the plasma membrane of thyrotrophs. This binding causes an activation of adenylate cyclase resulting in an increase of intracellular cyclic adenosine monophosphate (cAMP) accumulation, which ultimately leads to alteration of specific subcellular

functions and synthesis and release of TSH (19). TRH seems to have a modulating rather than a dynamic effect on the TSH secretion. There is now evidence that somatostatin and TRH do not act in a competitive manner at the receptor site of the thyrotroph, but that somatostatin suppresses the TRH action at a later stage (19). It has been shown that somatostatin inhibits cAMP accumulation *in vitro* in the pituitary, parallel to suppressing GH and TSH release (19). When the action of endogenous somatostatin is neutralized by passive immunization, its tonic suppression of cAMP accumulation in the thyrotrophs as well as in the somatotrophs may be eliminated, resulting in enhancement of the TSH response to TRH. Thyroid hormones exert their negative feedback effect on the TSH secretion at the pituitary level, without affecting to any major degree the TRH secretion (20). The action of thyroid hormones on the thyrotrophs has not been clarified yet, although there is some recent evidence that thyroid hormones reduce the number of TRH binding sites at the plasma membrane of the thyrotroph (19). The site of action may also be beyond the generation of cAMP in the cell nucleus (21, 22).

Administration of T₃ to rats naturally suppressed the basal TSH level and the TSH response to TRH in a dose-dependent manner. T₃ given at 100 ng/100 g BW suppressed the basal TSH level and blunted the response to TRH to about half the control level. Administration of the antiserum to somatostatin significantly increased the basal as well as post-TRH TSH levels as compared to the NSS-treated group. When 200 ng of T₃/100 g BW was administered, the antiserum to somatostatin did not alter the blocking effect of T₃ on the basal serum TSH, but still augmented the TRH-stimulated TSH secretion, as compared to the NSS-treatment. When greater doses of T₃ were given, the antiserum could not overcome the blocking effect of T₃ even after TRH. Vale *et al.* (6) have shown that somatostatin and thyroid hormones exhibit a summation of effects in inhibiting the TSH secretion both *in vivo* and *in vitro*. Our results are in good agreement with theirs, showing an interaction between somatosta-

tin and thyroid hormones under physiological conditions.

The rise of serum TSH after thyroidectomy was significantly increased by pretreatment with antiserum to somatostatin. However, on the seventh day after thyroidectomy, while the serum TSH level had possibly reached its maximum, it could not be further increased by passive immunization with somatostatin.

It has been shown that somatostatin only slightly reduces the basal TSH concentration in serum in both animals and man, but suppresses the response of TSH to TRH. The higher the TSH levels, the greater is the absolute reduction of TSH concentration caused by somatostatin (6-9, 23). There is also a positive correlation between the basal TSH levels and the net increment in serum TSH after TRH. Our results show a positive correlation between the TSH levels in the corresponding NSS-treated control groups and the net increase in TSH in anti-SS-treated groups.

Previous findings (14, 15) as well as those reported in this paper suggest that endogenous somatostatin may play an important role in regulating the TSH secretion, unless the circulating thyroid hormone concentration is extremely low or high. Thus, under normal conditions, somatostatin could be a physiological regulator of the TSH secretion, in addition to TRH and thyroid hormones. The role of somatostatin in regulation of the TSH secretion can, however, be completely evaluated only when we are able to measure fluctuations of endogenous somatostatin under various conditions.

Summary. Passive immunization of rats with antiserum to somatostatin (anti-SS) caused about a 2-fold increase in the basal serum TSH levels and a 1.5-fold rise in serum TSH after TRH, as compared to rats treated with normal sheep serum (NSS). When the basal and the TRH-stimulated TSH levels were suppressed to about half the control levels with 100 ng of T₃/100 g BW, the neutralization of the endogenous somatostatin secretion with anti-SS significantly increased the basal as well as the post-TRH TSH levels. After administration of 200 ng of T₃/100 g BW, the basal TSH levels in the anti-SS- and NSS-treated ani-

als were the same, but, in response to TRH, the TSH levels rose significantly higher in the anti-SS-treated group. When higher doses of T₃ (1 and 10 µg/100 g BW) were given, anti-SS failed to affect the basal or the post-TRH TSH levels. The elevated TSH levels 1 and 3 days after thyroidectomy were further increased by treatment with anti-SS as compared to NSS. The highly elevated level of TSH on the seventh day after thyroidectomy was, however, not affected by anti-SS. These findings indicate that somatostatin modulates the TSH secretion rate under physiological conditions and can be considered with TRH and thyroid hormones as another regulator of TSH secretion.

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Platelet Function Studies in Dogs with Cyclic Hematopoiesis¹ (39538)

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Cyclic neutropenia (CN) or cyclic hematopoiesis (CH) in man is characterized by regular attacks at 21-day intervals of fever, mucosal ulcerations, and neutropenia (1). The duration of the cycle remains constant for most blood elements, but the phase of the cycle or the onset of each element cycling is staggered (2). In 1967 a similar disease was reported in grey collie dogs (3), and the similarity of the disorder in man and dogs has now been cited in numerous reports (4-7). The disease is characterized in dogs as an autosomal recessive trait phenotypically expressed as a grey coat color. Cyclic neutropenia, as well as cycling of the other blood elements, occurs on an 11- to 12-day cycle in the collie and is accompanied by frequent infections during the neutropenic episodes. Since similar cellular fluctuations exist in both humans and dogs, a similar hematopoietic disorder has been hypothesized (8).

Most of the studies concerning cyclic hematopoiesis have dealt with leukocytes and erythrocytes, but not with platelets. Since all the blood cell types may exhibit a cyclic nature, one could hypothesize that blood platelets in CH dogs undergo a cyclic functional alteration with age as has been shown in a patient with thrombopoietin deficiency (9). Therefore, the purpose of this study was to determine if the cyclic nature of the peripherally circulating blood platelets resulted in an effect on platelet adhesion and platelet clot retraction.

Materials and methods. Platelet adhesion. Platelet adhesion or retention index was determined by the Salzman method (10).

Platelet clot retraction. Platelet clot retrac-

tion was determined according to the method outlined by McDonald *et al.* (11). Eight to ten milliliters of blood were withdrawn from the jugular vein into syringes containing 0.2 vol of a 1% Na₂-EDTA solution in 0.538% saline. The platelet-rich plasma (PRP) was separated from the other blood cells by centrifugation (200g) for 10 min at 5°. A 0.5-ml sample of PRP was removed and the remaining blood was centrifuged at 760g for 30 min to obtain the platelet-poor plasma (PPP). A Particle Data Celloscope was used to determine the number of platelets in suspension (1:2000 dilution in Isoton). Dilutions were made of PRP samples to final counts of 300,000, 150,000, 75,000, 37,500, 18,750, and 9,375 platelets/mm³ in a 0.2-ml sample of Na₂-EDTA-PPP. A 0.5-ml sample of the Na₂-EDTA-PPP containing a 0.2 vol of 0.33 M CaCl₂ was added to each of the six platelet dilutions for each dog; then bovine thrombin (0.05 ml containing 50 U/ml) was added to initiate clot formation. The final volume of 0.75 ml contained 1.88×10^6 to 60×10^6 platelets, 0.033 mmoles of Ca²⁺, 1.2 mg of Na₂-EDTA, and 2.5 U of thrombin. The samples were incubated in a 37° water bath for 10 min and the clots were separated from the walls of the tube by use of a small spatula. After a 2 hr incubation, the clots (along with trapped plasma) were weighed to determine the extent of retraction. The data were analyzed by linear regression with the platelet concentrations as the independent variable and the weights of the clots as the dependent variable.

Platelet clot retraction with platelet-plasma combination. In some experiments the plasma was separated from the platelets by centrifugation of the PRP (760g) at 5° for 15 min. The plasma from normal, CH, and transplant-CH dogs (CH dogs with normal marrow transplant) was combined with the platelets from normal, CH, and transplant-CH dogs to obtain the following mixtures of

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platelets and plasma: platelets from normal dogs with plasma from either normal, CH, or transplant-CH dogs; platelets from CH dogs with plasma from either normal, CH, or transplant-CH dogs; and platelets from transplant-CH dogs with plasma from either normal, CH, or transplant-CH dogs. Platelet clot retraction was then measured as described above.

Transplant technique. The bone marrow transplants were carried out as previously described (12, 13). Prior to transplantation the dogs used in these studies (normal dog No. 220 bearing a CH marrow graft, CH 309, and CH 310 bearing a normal marrow graft) were exposed to a lethal dose of irradiation (1250 R) over a 2-hr period.

Neutrophil staining and counting. The neutrophil counts were done by standard techniques as previously described (14). The data were expressed as the absolute neutrophil count, i.e., the percentage of neutrophils multiplied by the total leukocyte count.

Fibrinogen determinations. Fibrinogen determinations were made by use of a commercially available kit (Data-F Fibrinogen Determination, DADE Division, American Hospital Supply Corporation, Miami, Fla.). Bovine thrombin was added to a 1:10 dilution of fresh citrated plasma (diluted with Owen's Veronal buffer) and the clotting time was compared to that of a standardized fibrinogen preparation.

Results. Figure 1 illustrates the daily neutrophil counts of CH and normal dogs. The absolute neutrophil count for normal dogs varied from 5.3 to $7.3 \times 10^3/\text{mm}^3$, whereas the neutrophil counts in the CH dogs varied from $0.15 \times 10^3/\text{mm}^3$ during neutropenia (Days 7-8) to $17.3 \times 10^3/\text{mm}^3$ immediately after neutropenia. Generally, recovery from the neutropenia was accompanied by a severe leukocytosis only when infections were present.

Figure 2 shows daily platelet counts for CH dogs and the average platelet count for normal littermates. The platelets in CH dogs reach a peak immediately before neutropenia. The platelet cycle is, therefore, out of phase with the neutrophil cycle.

As shown in Fig. 3, the percentage of platelet adhesion was consistently depressed

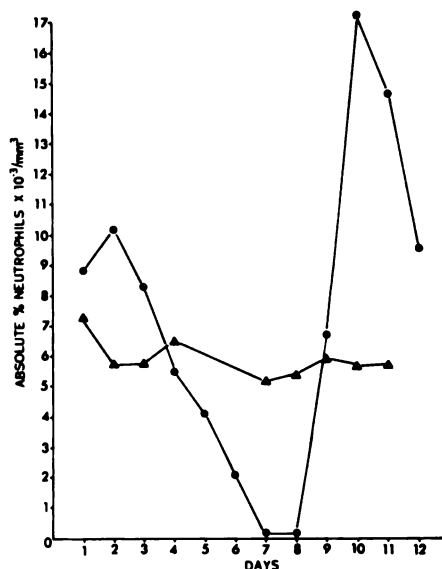


FIG. 1. Absolute percentages of neutrophil counts of normal (No. 358 and 360, ▲) and CH dogs (No. 346 and 181, ●) during one complete cycle.

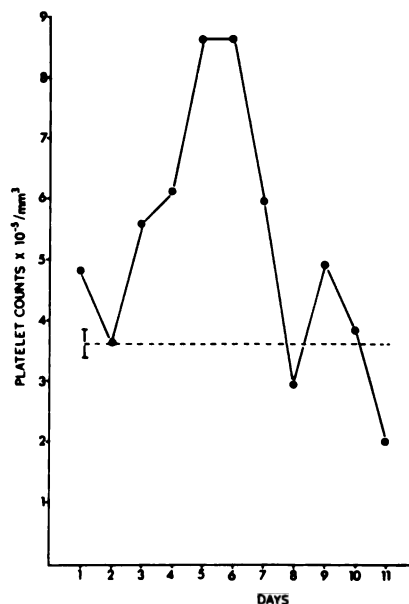


FIG. 2. Peripheral platelet counts of normal (No. 358 and 360, shown by horizontal line) and CH dogs (No. 346 and 181, ●) during one complete cycle.

in CH dogs as compared to normal controls. The percentage of adhesion of platelets from CH dogs remained below 32% during the entire neutrophil cycle. The results of

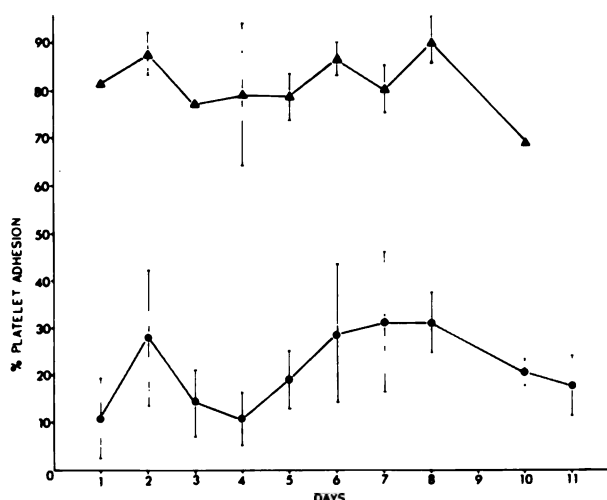


FIG. 3. Adhesion of platelets from normal (No. 359 and 360, ▲) and platelets from CH dogs (No. 361, 362, and 364, ●) during one complete cycle.

testing the percentage of adhesion of platelets from a CH dog and three dogs transplanted with bone marrow are shown in Table I. The platelet adhesiveness index was depressed to below normal (44%) in a normal dog transplanted with marrow from a CH dog. Conversely, the platelet adhesion values were normal (80–90%) in both CH dogs after transplantation with normal marrow. The CH dog without transplantation showed a low platelet adhesion in agreement with data for other dogs presented in Fig. 3.

Table II shows the least-squares analysis of the platelet clot retraction data obtained from normal and CH dogs over a 12-day period. The clot retraction of platelets from CH dogs was consistently less than that of platelets from normal dogs (the larger the intercept the larger the clot and the less functional are the platelets). As in the platelet adhesiveness studies, there was no relationship between platelet clot retraction data and the cyclic fluctuations of the platelets or neutrophils. The intercepts of the clot retraction were significantly ($P < 0.025$) different from one another, whereas the slopes were not.

Table III shows the results obtained from clot retraction studies after platelet-plasma combinations involving platelets and plasma from normal and CH dogs and a CH dog transplanted with normal marrow (trans-

TABLE I. EFFECT OF BONE MARROW TRANSPLANTATION ON PLATELET ADHESION.

Treatment	Adhesion (%)
Normal dog No. 220 transplanted with CH marrow	44
CH dog No. 310 transplanted with normal marrow	80
CH dog No. 309 transplanted with normal marrow	90
CH dog No. 338 (adult), no transplant	24

plant-CH). When platelets were incubated in normal plasma (a–c) there was no significant change in the intercept among the three groups (a–c). However, when one compares the intercepts obtained after incubation of platelets in normal plasma (a–c) with those in CH plasma (d–f), there is a significant ($P < 0.05$) increase in the values of the intercepts indicating less clot retraction or less functional platelets. When platelets were incubated with plasma from a transplant-CH dog (g–i), the intercepts were consistently decreased indicating an increased functional capacity perhaps due to the presence or absence of a factor in the transplant-CH dog's plasma.

Since the plasma from either normal or transplant-CH dogs corrected the clot retraction defect, the existence of an altered plasma factor was considered. Since fibrinogen levels are known to influence platelet

TABLE II. CLOT RETRACTION OF PLATELETS FROM NORMAL AND CH DOGS.

Treatment ^a	Number of dogs	Number of determinations	Least-squares analysis	
			Intercept \pm SE (weight of clot (mg))	Slope \pm SE (platelets $\times 10^{-3}/\text{mm}^3$)
Normal	2	72	2.27 ± 0.31	-5.91 ± 0.93
CH	2	90	3.36 ± 0.40^b	-8.23 ± 1.11

^a The dogs used in this study were as follows: two normal dogs No. 358 and 360 (9–11 months old); two CH dogs No. 181 (2½ years old) and No. 346 (10 months old).

^b Platelet clots from CH dogs were significantly larger than clots formed by platelets of normal dogs ($P < 0.05$).

TABLE III. CLOT RETRACTION EXPERIMENTS WITH VARIOUS PLATELET-PLASMA CONCENTRATIONS.

Treatment ^a		Number of determinations	Least-squares analysis	
Platelets	Plasma		Intercept \pm SE (weight of clot (mg))	Slope \pm SE (platelets $\times 10^{-3}/\text{mm}^3$)
a. Normal	Normal	24	1.44 ± 0.28	-4.62 ± 3.11
b. CH	Normal	24	1.50 ± 0.43	-2.40 ± 0.09
c. Transplant-CH	Normal	12	2.02 ± 0.10	-6.49 ± 0.17
d. Normal	CH	24	3.00 ± 0.86	-4.54 ± 10.72
e. CH	CH	12	3.29 ± 1.07	-2.92 ± 0.53
f. Transplant-CH	CH	12	3.15 ± 1.09	-6.07 ± 0.51
g. Normal	Transplant-CH	12	0.52 ± 0.00	-1.37 ± 0.00
h. CH	Transplant-CH	12	0.64 ± 0.01	-1.80 ± 0.43
i. Transplant-CH	Transplant-CH	12	0.69 ± 0.18	-1.58 ± 2.53

^a The dogs used in this study were as follows: two normal dogs No. 358 and 360 (9–11 months old); two CH dogs No. 181 (2½ years old) and No. 346 (10 months old); and No. 310, a CH dog that had received normal bone marrow 6–8 months prior to experimentation.

action, the concentration of this procoagulant protein was measured during one complete neutrophil cycle. Figure 4 shows the results of the fibrinogen determination in three dogs along with the absolute percentage neutrophil count. The mean values for the normal and transplant-CH dog are depicted for the purpose of illustration rather than the individual counts. In general, fibrinogen levels fluctuate in unison with neutrophil counts during the CH cycle. However, the fibrinogen values for the CH dogs are consistently elevated above normal and transplant-CH levels.

Discussion. The results of this study indicate that platelet adhesion and platelet clot retraction were consistently depressed in dogs with cyclic hematopoiesis (CH) and in single normal dog transplanted with CH marrow, whereas fibrinogen levels were elevated in the CH dogs. These decreased platelet functions and elevated fibrinogen levels were returned to normal levels after autologous bone marrow transplantation in normal donors.

TABLE IV. PLASMA FIBRINOGEN DETERMINATIONS FROM A NORMAL, CH, AND TRANSPLANTED DOGS.

Dog (No.)	Number of determinations	Fibrinogen (mg/dl \pm SE)
Normal (360)	6	208.0 ± 16.5
CH (346)	6	442.5 ± 42.7^a
Transplant ^b (310)	6	242.5 ± 10.1

^a Plasma fibrinogen levels were significantly higher than normal or transplant ($P < 0.001$).

^b CH dog transplanted with normal marrow.

There is no apparent explanation for the depressed platelet adhesiveness index in conjunction with the elevated levels of fibrinogen observed in the CH dogs. It does seem possible that the excess fibrinogen "coats" the platelet membrane, thereby covering up the active sites on the membrane which are necessary for adhesion to occur. Mason *et al.* (15) reported that platelet adhesion increases with increasing levels of fibrinogen plateauing after a certain concentration, which would appear contradictory to our findings. One possible explanation

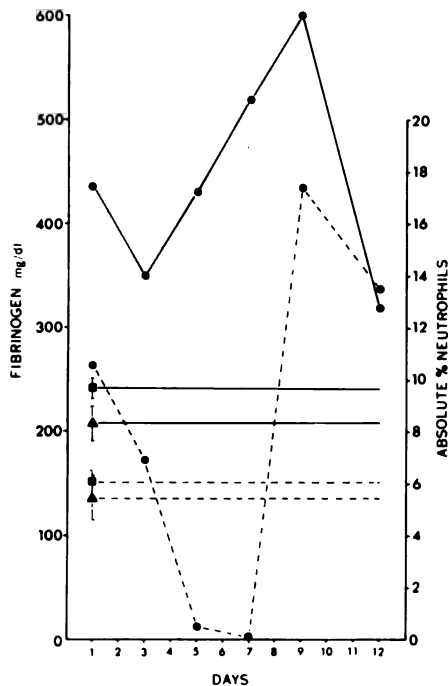


FIG. 4. Fibrinogen levels and absolute percentages of neutrophil counts of a normal (No. 358, ▲), a CH (No. 346, ●), and a transplant-CH (No. 310, ■) dog during one cycle. The fibrinogen and neutrophil values were presented as the mean \pm SE for illustrative purposes for the normal and transplant-CH dogs.

tion for the dichotomy could be an altered functional capability of the fibrinogen molecule in the CH dog, or perhaps the platelets themselves are congenitally deficient in CH animals. Another alternative hypothesis could be that platelet function and fibrinogen levels are not causally related to each other, though both are altered by the primary CH process. This seems to be the case in dogs affected by CH disease, especially in view of the findings expressed in Fig. 4.

Although the level of platelet adhesiveness is greatly reduced (Fig. 3), there does appear to be a slight increase in the percentage of adhesion on Days 5–8 following the phase of platelet production on Days 2–6 (Fig. 2). Since these dogs do not normally hemorrhage to death, platelets from CH dogs may be able to respond enough *in vivo* to maintain hemostasis. Perhaps the elevated levels of fibrinogen aid the platelets in their normal hemostatic role in CH dogs.

Depressed platelet clot retraction was also demonstrated in the CH dog. As with the platelet adhesiveness index, the ability of platelets to undergo normal clot retraction was restored after irradiation and reconstitution of CH dogs with normal bone marrow. In fact, the degree of clot retraction in dogs after transplantation was greater than in normal dogs.

Evidence for the existence of a plasma factor's influence on platelet clot retraction was found in platelet-plasma mixture experiments (Table III). Incubation of platelets from CH dogs in plasma from either normal or transplant-CH dogs resulted in normal clot retraction. Conversely, incubation of platelets from normal or transplant-CH dogs with plasma from CH dogs caused a decreased clot retraction.

These facts suggest that some plasma factor(s) is altered in the transplant recipient which enables the platelet function to return to normal limits. A previous study has shown that clot retraction can be altered by some plasma factor in rats (11). Rather than a lack of some plasma factor in the CH dog, an overabundance of a plasma component seemed to be responsible for the altered platelet function. Since plasma fibrinogen levels in CH dogs were consistently elevated over normal or transplant-CH levels, the hypothesis that fibrinogen is the contributory factor is conceivable. It should also be mentioned that fibrinogen levels are increased in dogs with infections such as lymphosarcoma, distemper, mild liver disease, and kidney disease (16). Therefore, the inflammatory changes that accompany CH of dogs (6) may account for the increased levels of fibrinogen. Reconstitution of CH dogs with normal marrow alleviates CH disease and infective processes and, thus, could account for the fibrinogen level's return to normal along with normal platelet adhesion and clot retraction.

Summary. Platelet adhesiveness, platelet clot retraction and fibrinogen determinations were made on dogs with cyclic hemato- poiesis (CH), dogs cured of CH disease via bone marrow transplantation, and normal dogs. Platelet adhesiveness and clot retraction values in CH dogs were below normal values throughout the cycle and were not

ed by neutropenic episodes. However, platelet functions were normal in dogs transplanted with normal marrow. Hemoglobin values of CH dogs did fluctuate with the neutrophil cycle, but were continued when compared to either normal dogs or CH dogs transplanted with normal marrow. By use of platelets and plasma from normal, CH, and transplant-CH dogs, we found that plasma from either normal or CH dogs transplanted with normal marrow corrected the CH dogs' platelet clotting defect. These studies indicate that a factor is involved in the decreased function of CH dogs; furthermore, this factor may be fibrinogen.

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Influence of Suckling on Gonadotropin Secretion in the Postpartum Rhesus Monkey¹ (39539)

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Although serum estradiol concentrations in lactating rhesus monkeys fall considerably below those observed during the follicular phase of the menstrual cycle, serum gonadotropin levels fail to rise (1). This finding suggests that suckling or its sequelae may override the negative feedback control system which governs tonic gonadotropin secretion (2). The present study was undertaken to investigate this phenomenon.

Materials and methods. Eight lactating monkeys were ovariectomized on Days 24–25 postpartum. Five of these were allowed to nurse their young while the infants of the remaining three were weaned at the time of surgery. Five of the foregoing animals were previously utilized for a study of the metabolic clearance rate of progesterone between Days 12 and 18 postpartum (3) but we have no reason to suspect that this procedure had any influence on the outcome of the present study.

Blood samples were taken by femoral venipuncture without sedation every other day from the time of parturition until termination of the study 30 days after ovariectomy. The sera were frozen until assayed for LH and FSH by heterologous radioimmunoassays described previously (4, 5) using rhesus LH (WDP-X-47BC, biopotency $1.7 \times$ NIH-LH-S1) and rhesus FSH (WDP-XI-93-4546, biopotency $14 \times$ NIH-FSH-S1) as

standards. The limits of sensitivity of these radioimmunoassays were 2–50 ng of LH/ml and 10–250 ng of FSH/ml. For purposes of calculation, gonadotropin levels which fell outside the limits of the assay were assigned the value of the limit. This practice distorts the data only in that it tends to minimize the differences between groups.

Anterior pituitary glands from lactating and cycling adult rhesus monkeys, kindly provided by Drs. P. Varavudho and C. S. Nicoll, were homogenized in a minimum of 4 vol of phosphate-buffered saline (pH 6.8). After centrifugation at 20,000g, the supernatants were assayed for FSH and LH by radioimmunoassay (4, 5). The postpartum age of the lactating animals from which the pituitary glands were removed is unknown.

Results. The effect of suckling on the secretion of gonadotropins in response to ovariectomy is shown in Fig. 1. Those animals which continued to suckle their young showed a marked retardation in the rise of serum LH concentrations after ovariectomy; in fact, no change in circulating LH was observed in four out of five animals in this group, the mean increase being attributable to but one monkey. In the animals which were weaned at the time of ovariectomy, serum LH concentrations rose to expected levels after a delay of 6–8 days. Similarly the postovariectomy rise in serum FSH concentration was also inhibited in the lactating animals but not quite as severely as that of LH (Fig. 1).

The LH content of pituitaries from lactating monkeys averaged less than 8% of that seen in normal cycling adults, while the FSH content varied from 38 to 98% of that of pregestational adult animals (Table I).

Discussion. These data suggest that the suckling stimulus and/or its sequelae inhibit pituitary LH secretion and, to a lesser extent, that of FSH. This effect is clearly independent of the ovary.

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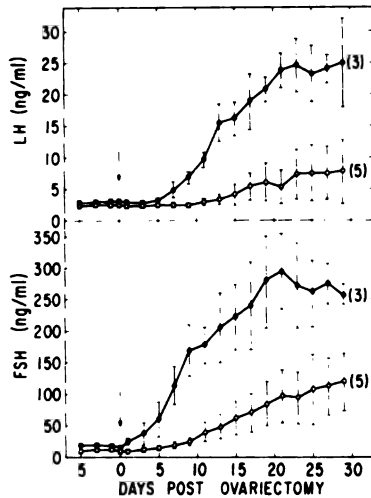


Fig. 1. Effect of suckling (○—○) on serum LH and FSH concentrations (mean \pm SE) following ovariectomy of lactating animals ($n = 5$) on 24–25 days postpartum (\downarrow). The control animals (●—●, $n = 3$) weaned at the time of ovariectomy.

TABLE 1. GONADOTROPIN CONTENT OF RHESUS MONKEY ANTERIOR PITUITARY GLANDS.

	LH (μ g/ gland)	FSH (μ g/ gland)
non-lactating females (pool of 20 glands)	119	295
lactating females (pool of 25 glands)	8	172
(pool of 20 glands)	<8	113
(pool of 5 glands)	9	290

The dichotomy between the FSH and LH responses to ovariectomy in the postpartum monkey, when taken together with the striking difference in pituitary content of LH in non-lactating and lactating monkeys, suggests that lactation may selectively inhibit pituitary gonadotropin synthesis. These studies in the monkey and previous work in the rat (6, 7) show that suckling inhibits gonadotropin production.

The divergence between LH and FSH patterns is similar to that seen in the monkey has also been observed in lactating women, where elevated prolactin levels are accompanied by constantly low serum LH, but rising FSH levels (8), suggesting an inverse functional relationship between prolactin and LH.

Whether prolactin plays a direct role in the control of gonadotropin secretion during lactation in the rhesus monkey awaits further study.

Of coincidental interest is the finding that both postpartum and prepubertal female rhesus monkeys show a delayed postcastration rise in gonadotropin levels and a low pituitary LH content coexistent with an FSH content characteristic of the adult cycling monkey (9).

Summary. The increase in serum gonadotropin concentrations which follows ovariectomy in the rhesus monkey is markedly retarded in lactating animals suckling their infants. This inhibitory influence of lactation is more pronounced for LH than FSH. The LH content of the pituitary glands removed from lactating monkeys is 8% of that found in pregestational adult females while the FSH content is not markedly reduced.

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Radioautography Related to Amounts of [^{35}S]Sulfate Taken up by Prostate Glands of 2-Year-Old Mice (39540)

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The dry weight of the solvent-extracted ventrolateral prostate gland of the 2-year-old is 1.5 times heavier than that of the 1-year-old strain A mouse (5.76 ± 1.84 and 3.78 ± 0.92 mg, respectively; $P = 0.0024$). This increase in the prostate gland occurs without any significant increase in the body weight of the mouse (33.8 ± 3.8 g for the 2-year-old and 33.4 ± 2.8 g for the 1-year-old; $P = 0.80$) (1). In addition, since old strain A mice could produce spontaneous adenocarcinoma of the prostate gland (2) and a suitable animal model is desirable, ^{35}S -uptake studies were performed (i) to determine whether the ventral and lateral prostatic lobes of the 2-year-old mice could take up ^{35}S injected intraperitoneally, and, if they could do so, (ii) to measure the amounts of ^{35}S in the lobes, (iii) to demonstrate radioautographically the ^{35}S localization in the prostatic tissue at predetermined times after ^{35}S injection, and (iv) to relate the relative ^{35}S distribution in the prostatic tissue with the amounts taken up by the prostate gland.

The studies above were based on nonsoluble ^{35}S , the ^{35}S retained by formalin-washed prostate glands. In order to obtain some relationship between the nonsoluble ^{35}S and the total prostate ^{35}S (nonsoluble plus soluble ^{35}S), the amounts of ^{35}S in the glands were measured before and after washing with formalin solution. Moreover, to obtain the relative amounts of ^{35}S in the blood of the prostate gland itself, the amounts of ^{35}S in the prostate glands were measured before and after perfusion with saline.

Materials and methods. Two-year-old, male, strain A mice (purchased from the Kirschbaum Memorial Lab, Baylor College of Medicine, Houston, Tex.) were injected intraperitoneally with dilute aqueous $\text{H}_2^{35}\text{SO}_4$ (carrier-free and HCl-free, purchased from New England Nuclear, Boston, Mass.) at a dose of $5 \mu\text{Ci/g}$ body weight.

The mice were individually anesthetized with diethyl ether and at 1, 5, 25, 50, and 125 hr after the injection, they were bled by cutting the inferior vena cava. Moistened with a few drops of saline, the lateral and ventral lobes of the prostate gland were freed from the prostatic capsule and excised (1). A dissecting microscope with a zoom attachment was used during the prostatectomy. The lobes were soaked immediately in 10% formalin solution containing 0.1% each of Na_2SO_4 and hexadecyltrimethylammonium bromide (Eastman Kodak Co.; practical grade was twice crystallized from acetone solution) and transferred into fresh solutions at regular intervals to remove soluble ^{35}S . Washing with the formalin solution was continued for 1 week, at which time the radioactivity of the wash was at background level. After blotting with gauze sponges, the washed prostate glands were dried to constant weight at 0.001 mm Hg, oxidized to a clear solution with a mixture of 0.1 ml of 70% HClO_4 and 0.2 ml of 30% H_2O_2 at 60° , cooled to room temperature, and then measured for radioactivity in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.) (3).

For radioautography, the prostate glands from four mice were excised at 10, 20, and 30 min and 1, 2, 5, 25, 50, and 125 hr after ^{35}S injection. After washing with the formalin solution described above, the glands from three of the four mice at each time point were dehydrated with ethanol, embedded in Epon-Araldite (4), cut at a thickness of $1 \mu\text{m}$, and mounted on glass slides.

The prostate gland of the fourth mouse at each time point was embedded in paraffin, cut serially at a thickness of $10 \mu\text{m}$, and placed on consecutively numbered glass slides. After deparaffinizing thoroughly with xylene and drying under reduced pressure, the even-numbered sections were kept

in methanolic HCl and the odd-numbered sections in absolute methanol at 37° for 16 hr (5, 6). Both groups were then washed with five changes of methanol and dried under reduced pressure.

The 1- and 10- μ m sections were coated with nuclear track emulsion NTB2 (Eastman Kodak Co.) by the dipping method, equilibrated at 40° and 80% humidity, dried for 1 hr at room temperature, sealed in light-proof boxes containing 8-mesh Drierite, and stored at 4°. At regular intervals of time, a set of these coated sections was developed in Dektol (7) and stained with hematoxylin and eosin (8) for microscopic studies.

Results. Both the ventral and lateral prostatic lobes of the 2-year-old mice took up intraperitoneally injected $^{35}\text{SO}_4$. The amounts of nonsoluble ^{35}S (not removable by washing with formalin solution) were the same for both lobes, at the various time points (Table I). Moreover, there was a sig-

nificant "peak" in the amounts of nonsoluble ^{35}S at 5 hr after the injection. At 25 hr, however, the amounts of ^{35}S had decreased to half the peak value; at 50 hr, to the 1-hr value; and at 125 hr, to less than the 1-hr value.

Table II shows that the injected ^{35}S appeared rapidly in the blood: The 1-hr blood samples had almost twice as much ^{35}S as the 5-hr samples. Once in the blood, the ^{35}S decreased relatively rapidly, from 26,400 cpm/mg at 1 hr to 59 cpm/mg at 125 hr. Similarly the ^{35}S of the nonperfused and saline-perfused prostate glands decreased from 1 to 125 hr. Nevertheless, there was a slight difference in the amounts of ^{35}S between the nonperfused and perfused glands, indicating the presence of small amounts of ^{35}S in the blood and large amounts in the tissue proper. In contrast to the unwashed glands (nonperfused and perfused), the formalin-washed glands showed a more gradual loss of ^{35}S . Consequently, the nonsoluble ^{35}S (represented by the formalin-washed glands) amounted to 1% of the total prostate ^{35}S (represented by the unwashed nonperfused and perfused glands) at 1 hr and to about 100% at 125 hr. Moreover, unlike the total prostate ^{35}S , the nonsoluble ^{35}S was greater in the 5-hr than in the 1-hr samples.

Radioautography showed that, at 10 min after its injection, ^{35}S was present in the epithelial cells and lumen of the acinus (Fig. 1A). At 1 hr, there was a relatively high concentration of ^{35}S in the epithelial cells with small amounts in the lumen (Fig. 1B). At 5 hr, there was a massive concentration

TABLE I. SPECIFIC RADIOACTIVITIES OF FORMALIN-WASHED VENTRAL AND LATERAL LOBES OF THE MOUSE PROSTATE GLANDS AT THE SPECIFIED TIMES AFTER $^{35}\text{SO}_4$ INJECTION.

Time (hr)	Ventral lobe ^a	P value	Laberal lobe ^a	P value
1	169 \pm 81		175 \pm 112	
5	436 \pm 68	0.069	484 \pm 86	0.089
25	241 \pm 58	0.016	242 \pm 62	0.015
50	167 \pm 49	0.15	213 \pm 34	0.53
125	105 \pm 51	0.23	98 \pm 31	0.018

^a Mean \pm SD. Each group consisted of five mice. Values are reported as counts per minute per milligram of dry weight.

TABLE II. SPECIFIC RADIOACTIVITIES OF BLOOD AND NONPERFUSED, SALINE-PERFUSED, AND FORMALIN-WASHED VENTROLATERAL PROSTATE GLANDS OF MICE AT THE SPECIFIED TIMES AFTER $^{35}\text{SO}_4$ INJECTION.

Time (hr)	Blood ^a	Prostate glands ^b		
		Nonperfused ^c	Saline-perfused ^d	Formalin-washed ^e
1	26,400 \pm 7,530	24,500	19,000	232
5	15,200 \pm 5,910	13,000	11,000	480
25	238 \pm 61	805	740	281
50	193 \pm 53	550	384	161
125	59 \pm 26	126	175	131

^a Mean \pm SD. Each group consisted of six mice. Values are reported as counts per minute per milligram of dry weight.

^b Average. Each group consisted of two mice.

^c Prostate glands excised from bled mice.

^d Prostate glands excised after perfusing with saline through the descending aorta.

^e Prostate glands (excised as nonperfused) thoroughly washed with formalin solution as described in the text.

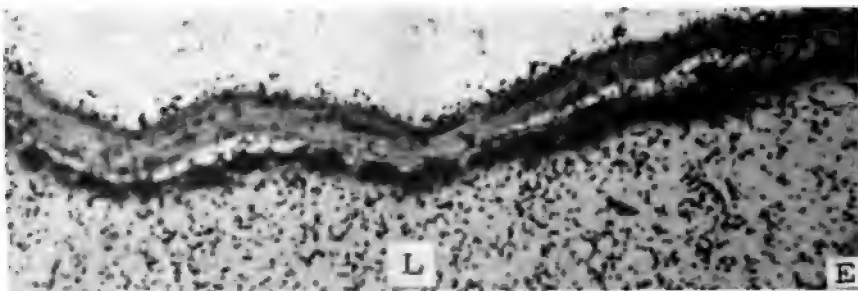
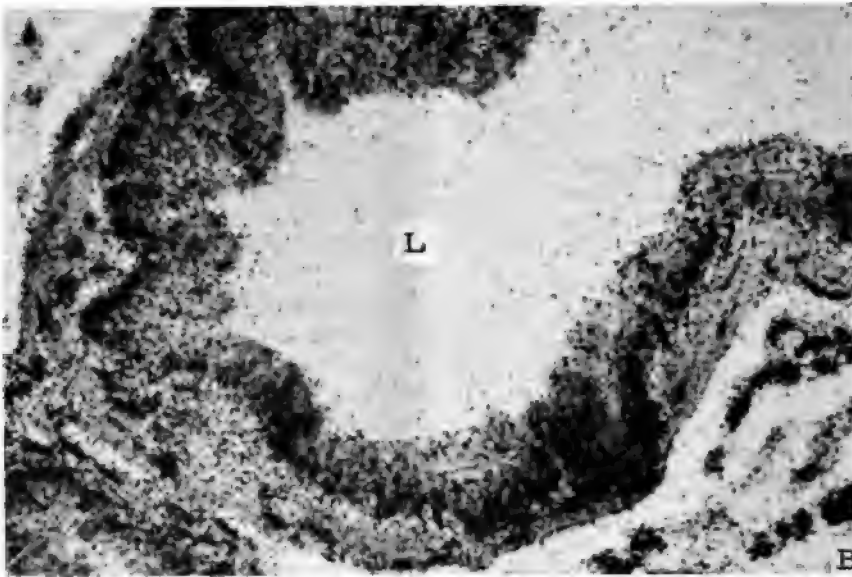
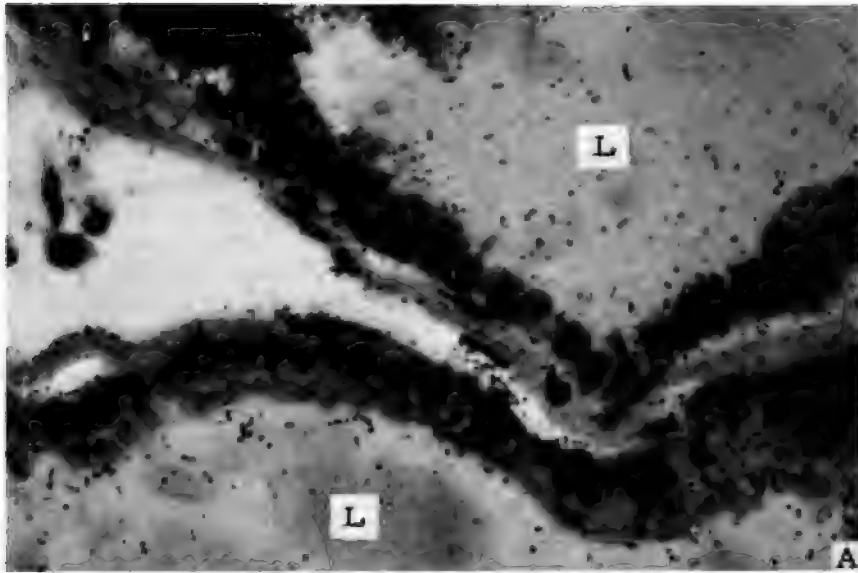


FIG. 1. Radioautographs of 1- μ m Epon-Araldite sections of ^{35}S -labeled mouse prostate glands developed simultaneously at 3 months of exposure and stained as described in the text. The glands were excised at 10 min (A), 1 hr (B), 5 hr (C), and 50 hr (D and E) after $^{35}\text{SO}_4$ injection. Photographed at 400 \times (A, B, and E), 250 \times (C), and 160 \times (D). L, lumen.

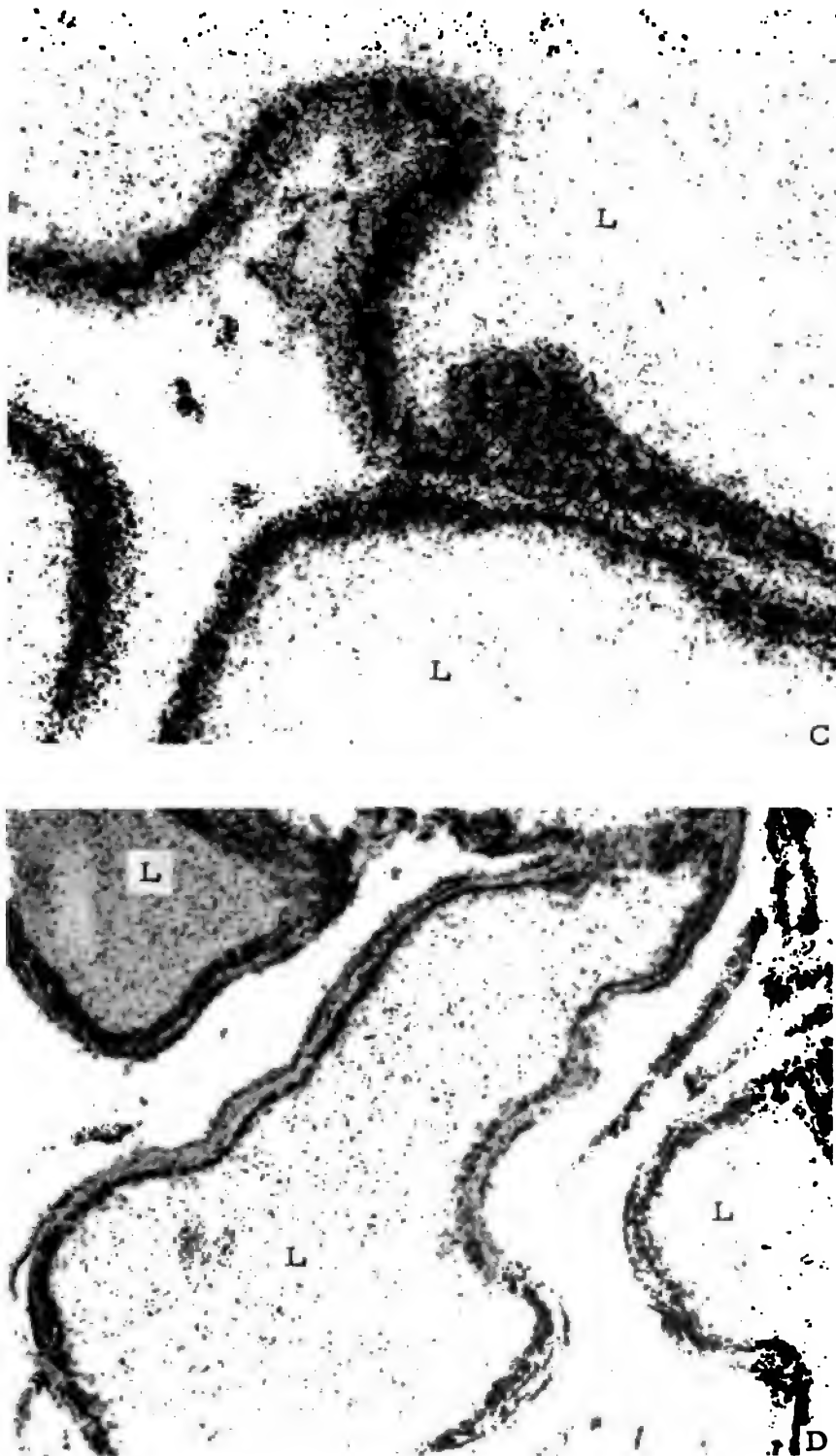


FIG. 1. (Continued)

of ^{35}S in the epithelial cells (Fig. 1C); however, at 50 hr, the amounts in the epithelial cells had decreased to the 10-min level while the amounts in the lumina had greatly increased (Fig. 1D). Marked differences in the amounts and distribution of ^{35}S were not observed between the 50- and 125-hr samples.

The radioautographs also showed that ^{35}S was taken up by connective tissue interstitial cells at the base of the prostatic acinar cells. However, the amounts of connective-tissue ^{35}S appear to be relatively small in comparison to those in the acinar cells at 10 min (Fig. 1A) and the acinar lumen at 50 hr (Fig. 1D).

Comparing Fig. 2A and 2B, it can be seen that the treatment of the prostatic tissues

with methanolic HCl reduced the number of silver grains.

Discussion. The radioautographs showed that prostate glands of the 2-year-old mice took up intraperitoneally injected ^{35}S (Fig. 1). The nonsoluble ^{35}S was initially concentrated in the epithelial cells of the glands and then moved into the lumina of the acini. This interpretation is based on the early appearance of ^{35}S in the epithelial cells (10 min after its injection, Fig. 1A), its relative increase in concentration from high (at 1 hr, Fig. 1B) to massive amounts (at 5 hr, Fig. 1C), and then to its decrease in the epithelial cells with a concomitant increase in the lumina (at 50 hr, Figs. 1D and E).

For comparative purposes, the ^{35}S -labeled prostate glands were processed for specific

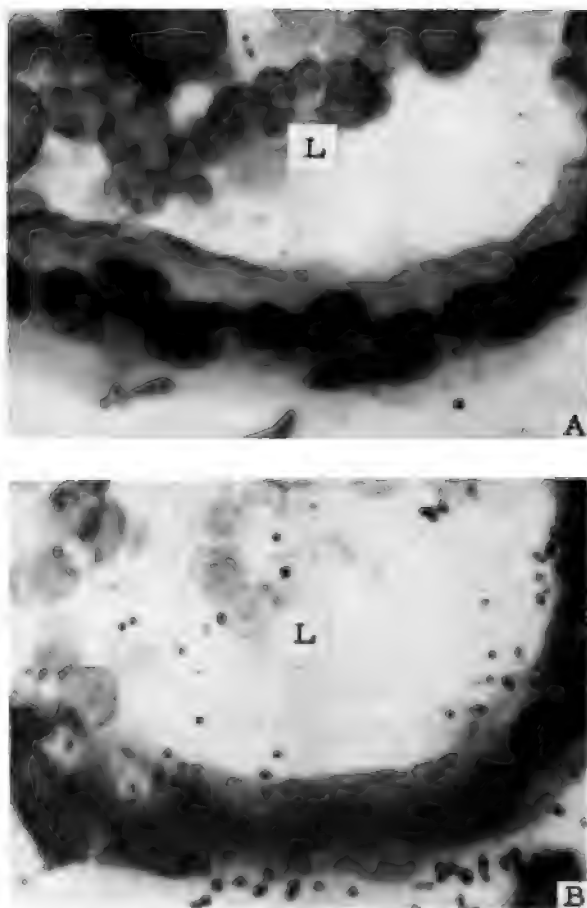


FIG. 2. Radioautographs of adjacent 10- μm paraffin sections of ^{35}S -labeled mouse prostate gland. (A). Treated with absolute methanol; (B), with methanolic HCl. Photographed at 400 \times . L, lumen.

activity determination (counts per milligram of dried glands; Table I) in the way as for radioautography. P calculated from the specific radioactivity of the prostate glands, showed a significant peak at 5 hr after ^{35}S injection. However, this peak appears not to be a true peak as one would expect the true peak to be beyond the 5-hr time point (the experiment was not designed to determine the peak).

At 25 hr, the specific radioactivity decreased to 55% of the peak; at 50 hr, to which was the same as the 1-hr time point. At 125 hr, the specific radioactivity decreased to 24% of the peak.

The radioautographs and specific radioactivity taken together led us to the following conclusion: The massive concentration of ^{35}S in the epithelial cells observed in the radioautograph (Fig. 1C) corresponded to the peak specific radioactivity of the 5-hr time point. After the 5-hr time point, the decrease in the specific radioactivities corresponded to the ^{35}S decrease in the epithelial cells and increase in the lumina (Fig. 1D). However, since the specific radioactivities at 1-hr (^{35}S predominantly in the epithelial cells, Fig. 1B) and the 50-hr (^{35}S predominantly in the lumina, Fig. 1D) time points were the same and amounted to only 24% of the peak, this discrepancy in the specific radioactivity of the 50-hr time point must be accounted for by normal loss of ^{35}S by excretion of the prostate glands. These results suggest that the prostate gland of the 2-year-old mouse is physiologically functional and capable of metabolizing $^{35}\text{SO}_4$.

It has been reported that only negligible amounts of administered $^{35}\text{SO}_4$ are incorporated into sulfur-containing amino acids (9). However, the nonsoluble ^{35}S in rat rib cartilage, rat bone cartilage (10), and rabbit cartilage (11) is incorporated almost exclusively into chondroitin sulfate. More direct exchange of $^{35}\text{SO}_4$ with the sulfolysosomal glycosaminoglycans (GAGs) appears to occur (12). We have shown that the soluble ^{35}S in formalin-washed prostatic glands are removable by treatment with aqueous HCl but not with absolute methanol (Fig. 2), just as the ester sulfate of GAGs are (6). Presumably, the water-, benzene-, xylene-, and methanol-insoluble

^{35}S (which is demonstrable radioautographically to be localized in specific areas of the prostatic tissues at specified times after ^{35}S injection) is an integral component of GAGs. If this is so, the secretion of the normal mouse prostate gland should contain sulfated GAGs (Fig. 1). However, it may be difficult to demonstrate their presence histochemically. Although the presence of GAGs in the luminal contents of carcinomatous prostate glands could be demonstrated, GAGs in normal human prostate glands could not be demonstrated with GAG stains (13, 14). Possibly, the GAGs of the normal gland are bound in such a way as to render them nonstainable with the usual methods (15). Nevertheless, various sulfated GAGs have been extracted from normal human prostate glands and those GAGs have been characterized and quantitated (16).

Summary. Intraperitoneally injected $^{35}\text{SO}_4$ appeared rapidly in the blood and in the prostate glands of the 2-year-old mouse. Most of the prostate ^{35}S was in the tissue proper with small amounts in the blood. The amounts of soluble ^{35}S of prostate glands were greatest at 1 hr and least at 125 hr after ^{35}S injection. The amounts of nonsoluble ^{35}S (not removable by washing with formalin solution) decreased much more gradually than the soluble ^{35}S . Consequently, the nonsoluble ^{35}S amounted to 1% of the total prostate ^{35}S at 1 hr and to about 100% at 125 hr.

The amounts of nonsoluble ^{35}S were the same for both the ventral and lateral prostatic lobes at the various time points after the injection. The nonsoluble ^{35}S increased to a peak at 5 hr, decreased to half the peak at 25 hr, and it continued to decrease with time.

At 10 min after its injection, ^{35}S was detectable in the epithelial cells and lumen of the acinus by radioautography. The amounts of ^{35}S in the epithelial cells increased from relatively large at 1 hr to massive amounts at 5 hr. However, at 50 hr, the ^{35}S in the epithelial cells had decreased to the 10-min level while the amounts in the lumina had greatly increased.

Results of this investigation suggest that the nonsoluble prostate ^{35}S was initially concentrated in the acinar cells, moved into the

lumen, and then was excreted by the prostate gland. Thus, the prostate gland of the 2-year-old mouse is physiologically active and capable of metabolizing $^{35}\text{SO}_4$.

The water-, ethanol-, xylene-, and methanol-insoluble ^{35}S of the prostate gland appears to be an integral component of GAGs.

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Relationship between Canine Factor VIII Coagulant Activity and Factor VIII-Related Antigen¹ (39541)

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Factor VIII (FVIII), the plasma component functionally defective in dogs with hemophilia A (1), can be isolated by gel electrophoresis in physiological buffer systems as a macromolecule with a molecular weight in excess of 10^6 daltons (2). The coagulant activity of canine FVIII (VIII-C) is characterized by its ability to correct the bleeding defect of hemophilia A plasma (1). In addition, canine FVIII gives a precipitin reaction against a specific heterologous antigen, and FVIII-related antigen (VIII-RA) is its non-coagulant property of canine FVIII is its ability to correct the defective ristocetin-induced platelet aggregation of plasma from dogs with von Willebrand's disease (4). Human and bovine FVIII exhibit all of these same characteristics (5-7).

Since all three of these properties are associated with the same chromatographic fraction on gel filtration (8, 9), it is possible that they represent three functions of a single molecule. However, recent studies have indicated that, as with human FVIII, canine FVIII-C and FVIII-RA can be segregated by high-salt-concentration ion exchange chromatography (2). By preparing heterologous antisera against different components of the FVIII complex and coupling the antisera to a solid-phase matrix, we have begun to study further whether FVIII is composed of more than one molecule. Differential removal of either FVIII-C or FVIII-RA from canine plasma by the insolubilized antisera would suggest that they are discrete molecules. A preliminary summary of the results described here has been reported (10).

Materials. Canine factor VIII was prepared

from the cryoprecipitate of 40 ml of plasma resuspended in 5 ml of 0.03 M barbital-buffered saline, pH 7.4, at a final concentration of 25 mg/ml. The crude FVIII preparation was chromatographed on a 2.5×43 -cm 4% agarose column (Bio-Gel A-15m, Bio-Rad Laboratories, Richmond, Calif.), and eluted with the resuspension buffer at 4° and 20 ml/hr. The 5-ml fractions were monitored for FVIII-C by the one-stage partial thromboplastin time method using canine FVIII-deficient substrate (11), and for FVIII-RA as previously described (3). The two fractions that eluted at the end of the void volume and contained 70 µg of protein/ml and the maximum amount of FVIII-C were pooled and stored in 1-ml aliquots at -40°.

A 4-kg crossbred Flemish giant-chinchilla rabbit was immunized with the gel-filtered canine FVIII preparation. The rabbit received eight weekly 0.5-ml intradermal injections of the FVIII preparation containing 2.5 to 5 units of FVIII (1 unit represents the FVIII-C or FVIII-RA level in 1 ml of a pool of equal volumes of plasma from eight randomly selected adult dogs, four of each sex) and 35 µg of protein mixed with an equal volume of sterile 0.23% $\text{Al}(\text{OH})_3$. The animal was rested for 1 month, reimmunized once, and bled 10 days later. The serum obtained was first decomplexed by heating for 30 min at 56° and then absorbed with 10 mg $\text{Ca}_3\text{PO}_4/\text{ml}$ at 25° for 20 min.

Canine hemophilia A plasma obtained from dogs in the Chapel Hill colony (1) was fractionated by the same cryoprecipitation and gel-filtration techniques. The hemophilia A plasma contained <0.01 U of FVIII-C/ml and >1.0 U of FVIII-RA/ml. The two chromatographic fractions that eluted at the end of the void volume and contained the maximum FVIII-RA were pooled, aliquoted, and used to raise a rabbit antiserum as above.

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The anti-normal (AN) and anti-hemophilia A (AH) antisera were analyzed by immunoelectrophoresis in 0.9% agarose and rendered monospecific by absorption with fractions of normal canine plasma made low in VIII-C and VIII-RA by the method of Zimmerman *et al.* (12). The specificities of the absorbed antisera were compared by Ouchterlony immunodiffusion. An aliquot of the AN antiserum was absorbed once with an 8% ethanol concentrate of the hemophilia A plasma to produce a third type of antiserum (Hem-AN), which neutralized VIII-C but was devoid of precipitating antibodies. The ratio of absorbing hemophilic plasma to antiserum was 0.5 to 1.0 vol.

The avidities of the AN and AH antisera for VIII-RA were determined by comparing electroimmunodiffusion peak heights produced with constant antigen concentrations and varying antisera dilutions, as described by Kernoff and Rizza (13). The precipitating characteristics of these antisera were also compared by repeatedly using each to determine the VIII-RA levels of plasma from a normal dog and from dogs with hemophilia A and von Willebrand's disease.

The patterns generated by the antisera on crossed immunoelectrophoresis were used to characterize further their precipitating similarities. Each gel was prepared on a 95-mm² glass plate. One-half consisted of 0.9% Seakem agarose; the rest contained the same gel plus monospecific rabbit anti-canine FVIII. A 3-mm well was punched in the nonimmunogel 1 mm from the boundary between the two halves. Six to ten microliters of an 8% ethanol concentrate of plasma were loaded in the well and electrophoresed in the Gelman Deluxe electrophoresis chamber at 15 mA for 2.5 hr in a path parallel to the gel interface, then for an additional 3.0 hr into the immunogel at 25°.

The anti-VIII-C activities of the antisera were determined by a modification of the second stage of the inhibitor neutralization method of Denson (14). Equal volumes (0.25 ml) of pooled normal canine platelet-poor plasma (containing 1 U of VIII-C/ml) and dilutions of the antisera were incubated for 2 hr at 37°, then centrifuged for 15 min at 700g. The supernatant was assayed for residual AHF. The inhibitor unit is defined

as the amount of inhibitor which will destroy 75% of the added factor VIII after a 2-hr incubation at 37°.

The globulin fractions of the monospecific antisera and nonimmune rabbit serum were isolated by half-saturation (NH₄)₂SO₄ precipitation, equilibrated in 0.05 M phosphate-buffered saline (pH 7.4), and concentrated against polyethylene glycol 20,000 to contain approximately 50 mg of protein/ml. Eighty milligrams of globulin from each antiserum and nonimmune rabbit serum were bound to 20 ml of packed Sepharose 2B beads activated with CNBr by the method of Cuatrecasas *et al.* (15). Unbound sites on the beads were blocked with 1.0 M glycine, followed by the standard acetate, urea, and NaHCO₃ washes. The antibody-coated beads were suspended to a 50% concentration in imidazole-buffered saline, pH 7.4.

The effect of the antibody and control beads on canine plasma was determined by mixing 0.60 ml of various combinations of nonimmune and antibody-coated beads with an equal volume of normal canine plasma in a 10 × 75-mm siliconized glass tube. After a 3-hr incubation at 37°, during which the tubes were inverted at 5-min intervals, the beads were separated from the plasma by centrifugation for 10 min at 700g. The supernatant was aspirated and assayed immediately for VIII-C and subsequently for VIII-RA by electroimmunoassay. Serial dilutions of the normal canine plasma, similarly incubated, were used as reference standards for both the coagulation and precipitation assays. The levels of VIII-C and VIII-RA after incubation with nonimmune beads were used as the control values in computing the percentage of residual VIII-C and VIII-RA.

Results. The absorbed AN and AH sera formed only one precipitin line when analyzed by immunoelectrophoresis, lines of complete identity by immunodiffusion (Fig. 1), and similar double-component precipitin patterns when analyzed by crossed immunoelectrophoresis. On electroimmunodiffusion 16% more AH antiserum was required to produce peak heights equal to those obtained with AN antiserum tested against the same normal canine plasma. Multiple VIII-RA determinations on three different canine plasma samples with both AN and AH

antisera gave values that were not statistically different ($P > 0.2$).

The absorbed AN antiserum contained 60 anti-VIII-C inhibitor units. No anti-VIII-C activity could be demonstrated by repeated neutralization assays using either the absorbed or nonabsorbed AH antiserum. When the AN antiserum was absorbed with hemophilia A plasma its precipitating avidity could not be detected by either Ouchterlony or Laurell methods, and its VIII-C neutralizing titer was decreased to 4 inhibitor units.

Thus, three types of anti-FVIII antisera were produced: (i) AN, which both neutralized VIII-C and precipitated VIII-RA; (ii) AH, which only precipitated VIII-RA; and (iii) Hem-AN, which only neutralized VIII-C.

The data from the three types of antibody-bead incubations are summarized in Table I. AN-globulin-coated beads at each of three dilutions removed similar amounts of VIII-C and VIII-RA from normal canine plasma. The same was true of AH-globulin-coated beads. In contrast, plasma incubated with 100% Hem-AN-coated beads retained 75.2% of VIII-RA but only 38.4% of the

VIII-C. The VIII-C and VIII-RA levels of plasma incubated with the control beads were altered only to the levels predicted as the effects of dilution.

Discussion. The precipitating antigen detected by the monospecific AN antiserum was considered to be analogous to human VIII-RA because of the antiserum detected: (i) no precipitin material in plasma from a patient homozygous for von Willebrand's disease and normal levels in healthy humans (H. R. Gralnick, personal communication); (ii) decreased levels of precipitin material in dogs from families with well-characterized von Willebrand's disease (3); (iii) elevated levels of precipitin material in dogs with hemophilia A (3); and (iv) precipitin material only in the void volume fractions of gel-filtered cryoprecipitates.

The precipitating material detected by the cross-reaction of AH antiserum with normal canine plasma appeared to be the same as that detected by AN antiserum on electroimmunoassay, double diffusion, and crossed immunoelectrophoresis. It was, therefore, also considered to be VIII-RA. The precipitating capacity of the AN antiserum was apparently abolished by its absorption with hemophilia A plasma, which left only the VIII-C neutralizing capacity.

Two of the three antibody systems appeared to remove VIII-RA and VIII-C simultaneously. Undiluted beads coated with the globulin fraction of an antiserum that precipitates VIII-RA but has no neutralizing capacity (AH-coated beads) removed about the same amount of VIII-C and VIII-RA from normal canine plasma, as did AN-coated beads, which have both anti-

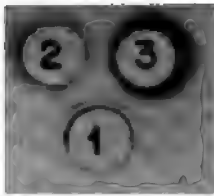


FIG. 1. Agar gel immunodiffusion. The wells contained: 1, normal canine plasma; 2, anti-normal canine FVIII; 3, anti-hemophilic canine FVIII.

TABLE I. VIII-RA AND VIII-C (%) IN NORMAL CANINE PLASMA AFTER INCUBATION WITH ANTI-FVIII-COATED SEPHAROSE BEADS.^a

Antibody-coated beads (%) ^b	Antibody coated to beads		
	Anti-normal FVIII	Anti-hemophilic FVIII	Hemophilic-absorbed antinormal FVIII
100.0 FVIII-RA	28.1 ± 13.0	39.8 ± 12.1	75.2 ± 8.3
FVIII-C	32.7 ± 16.0	35.3 ± 16.1	38.4 ± 8.5
33.3 FVIII-RA	44.4 ± 17.3	69.0 ± 18.2	84.5 ± 11.7
FVIII-C	63.7 ± 23.2	82.2 ± 14.4	61.0 ± 15.4
16.5 FVIII-RA	67.0 ± 24.7	79.0 ± 9.6	106 ± 28.0
FVIII-C	86.3 ± 16.2	95.6 ± 5.1	74.2 ± 12.4

^a Mean ± SD; $n = 6$. Each determination is the quotient of the final experimental value and control level determined with nonimmune globulin beads.

^b Percentage of anti-FVIII-coated beads mixed with control globulin beads.

VIII-C and anti-VIII-RA bound antibodies. These results, especially those with AH-coated beads, suggest that VIII-RA and VIII-C may reside on a single molecule or a linked complex.

The removal of Hem-AN-coated beads of about twice as much VIII-C as VIII-RA is inconsistent with the above hypothesis. We feel justified, however, in placing less emphasis on these findings because production of the Hem-AN antibody required additional manipulation which might have influenced the results. It is also difficult to resolve how Hem-AN antiserum, which contains only 4 VIII-C inhibitor units/ml, can remove VIII-C as effectively as AN antiserum, which contains 60 inhibitor units/ml.

The concept that human VIII-C and VIII-RA are separate entities has received support from studies reported by Zimmerman and Edgington (16), who used a solid-phase antibody system, and by Hougie *et al.* (17), who used a double antibody technique. These results are in contrast to those reported in this study as well as to those of Hoyer (18) and Bird and Rizza (19), who have proposed that VIII-C and VIII-RA are part of the same molecule or complex because immunoprecipitates containing VIII-RA also contain VIII-C. Jaffé and Nachman (20) have suggested that all FVIII molecules contain VIII-RA, whereas a small percentage contain VIII-C.

Others have demonstrated that human VIII-C and VIII-RA can be physically separated by gel filtration (21), ion-exchange chromatography (22), and cryoprecipitation (23). In addition, it has been well documented that VIII-C is generated independently of VIII-RA after infusion of FVIII preparations into human patients and dogs with von Willebrand's disease (24-26; Bouma, Dodds, van Mourik, Sixma, and Webster, unpublished). These reports all demonstrate that under appropriate conditions, VIII-C and VIII-RA can be separated; however, there is still a lack of consensus about the physical relationship of circulating VIII-C and VIII-RA.

Our earlier studies (27) suggested that canine VIII-C and VIII-RA represent different antigenic sites on the canine FVIII complex but no speculation was made about their physical relationship. Most of the data

presented in this study support the concept that *in vivo* VIII-C and VIII-RA may behave as a single macromolecule or complex.

Summary. Two of three insolubilized anti-canine FVIII systems removed similar amounts of VIII-C and VIII-RA from canine plasma. These data suggest that VIII-C and VIII-RA form part of one circulating complex.

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Glucagon from Avian Pancreatic Islets: Purification and Partial Characterization of a 9000-Dalton Species with Glucagon Immunoreactivity^{1,2} (39542)

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Studies of glucagon biosynthesis have shown that labeled amino acids are incorporated into islet proteins ranging in molecular weight from 69,000 to 6000 daltons and, therefore, are larger than glucagon itself (3500 daltons (2-8)). Although these proteins may represent proglucagon molecules, relatively little has been done to purify them for biochemical characterization. Using isolated pigeon islets, we observed the incorporation of ³H-labeled tryptophan into a 69,000-dalton (Peak I) and a 9000-dalton component (Peak II) (5, 6). Both fractions reacted with glucagon antisera (AGS), and Peak II was significantly more immunoreactive than Peak I. Immunoreactive forms of similar sizes have been found in extracts of canine pancreas and mouse islets (9) and in human plasma (10). This paper describes additional attempts to extract and purify proteins with glucagon immunoreactivity from pigeon islets.

Methods and materials. Islet preparation. Pigeons (Mogul Ed Company, Oshkosh, Wis.), fasted for a week before the experiments, were sacrificed with an overdose of sodium pentobarbital. The islets were prepared using a modification of the method of Lacy and Kostianovsky (11) as follows: After disrupting the exocrine tissue with Hanks' buffer (12) injected into the pancreatic ducts, the pancreata were removed and cut in half, and each half was placed in a glass vial containing 0.1 ml of a collagenase

solution (Type III, Sigma Chemical Co., St. Louis, Mo., 0.4 mg/ml). The tissue was minced for 10 min, shaken for 10 min at 37°, and minced for an additional 10 min. Minced samples from five or six birds were pooled, suspended in 250 ml of ice-cold Hanks' buffer in a 500-ml beaker, and further disrupted by repeated passage through a 30-ml glass syringe (20 times without a needle and 15 times with a No. 15 needle). The beaker containing the suspension was allowed to rest on ice for 2 min, and the islet-containing supernatant was decanted and placed on ice for 45 min. The islets were removed from the bottom of the beaker by means of a Pasteur pipet and placed in a test tube. An aliquot of about 0.2 ml of the islet suspension was removed with a Pasteur pipet and was replaced by an equal volume of Hanks' buffer, and the suspension was again stirred vigorously and allowed to stand 2 min before removing the next aliquot. Islets collected within a 45-min period were centrifuged at 200 rpm for 1 min using a table-top clinical centrifuge. The pellet was washed six times with 10 ml of ice-cold Hanks' buffer. Trichloroacetic acid (TCA, 30%, w/v) was added to the islet suspension to a final concentration of 15%. The suspension was centrifuged at 2000 rpm for 3 min. The TCA-precipitated islets were stored at -20° for subsequent extraction with acid ethanol (13). Alternately, the islets were treated with a mixture of 1 ml of 30% TCA and 1 ml of 1 mM benzamidine hydrochloride (Aldrich Chemicals, Milwaukee, Wis.) in 1 M acetic acid or with 2 ml of 15% TCA containing 500 kiu of aprotinin solution (Trasylol, FBA Pharmaceuticals, Inc., New York, N.Y.). These proteolytic enzyme inhibitors were added to reduce the degradation of glucagon-related proteins during the purification procedure (14). Since aprotinin, a 6000- to 7000-dalton protein, would have interfered with the chro-

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raphic procedures while benzamidine chloride can be removed by dialysis or gel filtration, we used the latter for the preparations described in this

Fractionation of islet proteins and gel filtration. CA-precipitated islets were homogenized in acid ethanol using a Potter-Elvehjem homogenizer (13) and extracted by stirring overnight at 4°. In a typical large-scale experiment, islets from 20 birds were extracted in 40 ml of acid ethanol containing 1 mM benzamidine hydrochloride and 1 M acetic acid. The acid alcohol-soluble fractions, obtained by centrifuging homogenate at 2000 rpm for 15 min in a Sorvall centrifuge, were lyophilized, reconstituted in acetic acid, and chromatographed on a Sephadex G-50 column (Pharmacia Fine Chemicals, Piscataway, N.J.) that had been equilibrated in 1 M acetic acid.

Absorbance at 275 nm was determined using a Gilford uv spectrophotometer. Aliquots were used for glucagon radioimmunoassay. Appropriate fractions were pooled, lyophilized in the presence of benzamidine-HCl, and stored at -20° for further fractionations.

Ion-exchange column chromatography. Diethylaminoethyl (DEAE)-cellulose (DE-65, Whatman Co., supplied by Reeve Analytical, N.J.), equilibrated in 0.01 M HCl, pH 8.7, followed by 0.01 M Tris-3 M urea (charcoal-treated) (Sigma Chemical Co., St. Louis, Mo.), was packed in 5 × 0.90-cm columns (Pharmacia Fine Chemicals, Piscataway, N.J.), which had been washed with Tris-HCl-urea buffer for

A 2-ml sample of protein solution in Tris-HCl-urea was applied to the column and eluted with 20 ml of the buffer containing 0.01 M NaCl, followed by 20 ml of the buffer containing 0.1 M NaCl. Fractions of 1 ml were collected. Urea and salt were removed by dialyzing the fractions at 4° through three changes of distilled water (3 × 48 hr each), using Spectrapor membranes (Spectrum Medical Industries, Inc., Los Angeles, Calif.) with a molecular weight cutoff of 3500 daltons. The dialyzed material was lyophilized and stored at -20° for further analysis.

Polyacrylamide-gel electrophoresis. Poly-

acrylamide-gel electrophoresis was performed at pH 3.5 using 7.5% gels according to Moller and Chrambach (16). The dimensions of the gel were 7 × 75 mm; running time was 10 hr at 5 mA/gel. The gels were stained for protein with amido black and destained in 7% acetic acid.

Amino acid analysis. Protein fractions were hydrolyzed in 6 N HCl and amino acid analyses were performed by L. H. Ericson of the AAA Laboratory, Seattle, Wash., using a Durrum analyzer, Model D-500. Two separate samples were analyzed.

Radioimmunoassay for glucagon. Radioimmunoassay for glucagon was performed using a method previously described (17), except that dextran-coated charcoal was used to separate free from antibody-bound hormone (18). Aliquots of 0.2 ml from consecutive gel-filtration fractions were pooled and lyophilized with 2000 kiu of aprotinin, redissolved in 0.7 ml of sodium barbital-albumin buffer, pH 8.7, cleared by centrifugation, and assayed for glucagon.

Aliquots of 0.1 ml, from ion-exchange fractions, were dialyzed against distilled water in the presence of 1000 kiu of aprotinin, lyophilized, and dissolved in 0.5 ml of sodium barbital-albumin buffer for radioimmunoassay.

Results. Fractionation of acid ethanol-soluble islet proteins with a Sephadex G-50 (fine) column yielded three peaks with absorbance at 275 nm (Fig. 1A). Peak I eluted at the void volume, Peak II eluted as a component of ≈9000 daltons, and Peak V eluted at the salt volume. Radioimmunoassay indicated that the bulk of the immunoreactive material was located in the glucagon region (III and IV), with smaller amounts eluting as Peaks I and II (Fig. 1B). Fractions corresponding to Peak II were pooled, lyophilized, reconstituted in 1 M acetic acid, and rechromatographed on a Sephadex G-50 column, eluted with 1 M acetic acid. Figures 2A and B show that, upon rechromatography, Peak II appears as a major immunoreactive component. In addition, protein components with glucagon immunoreactivity eluted in the glucagon region and beyond. However, when islets were extracted in the presence of inhibitors of proteolysis, more than 90% of the immu-

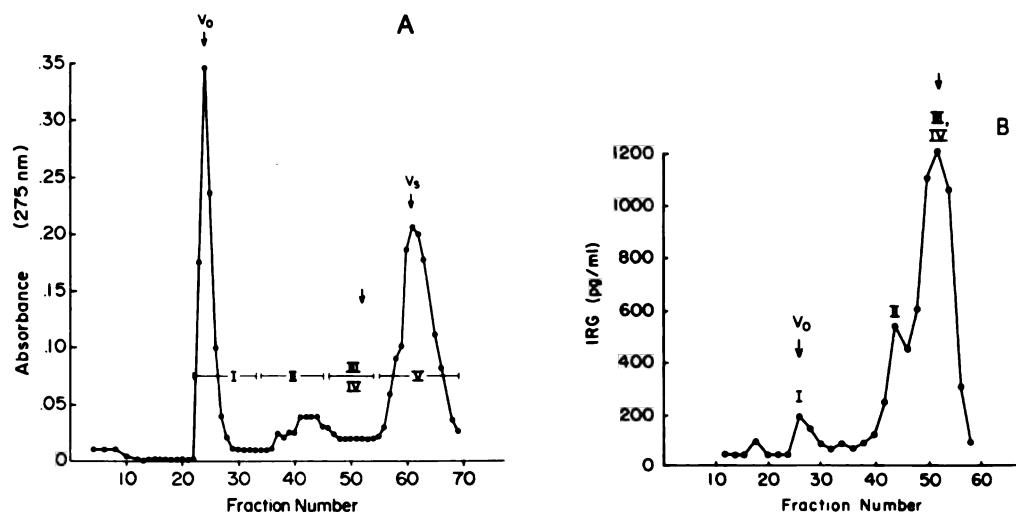


FIG. 1. (A) Sephadex G-50 gel filtration of acid ethanol-soluble islet proteins. Column size, 80×2.5 . Fraction volume, 2 ml. TCA-treated islets from 16 birds were extracted with acid ethanol. The soluble fraction lyophilized, taken up in 5 ml of 1 M acetic acid and 0.5 ml of glacial acetic acid and chromatographed. V_0 , void volume. V_s , salt volume. \downarrow , position of ^{125}I -labeled porcine glucagon. (B) Aliquots of 0.2 ml from consecutive fractions were pooled and lyophilized with 2000 kiu of aprotinin. Each lyophilized fraction was dissolved in 0.1 ml of sodium barbital-albumin buffer, pH 8.7, insoluble materials were removed by centrifugation and the supernatant was assayed for glucagon. IRG, concentration of immunoreactive glucagon per assay sample.

noreactivity was eluted as Peak II (Fig. 2C). These results suggest that Peak II was degraded by proteases present in the extracts, resulting in products immunologically related to glucagon.

Fractions corresponding to Peak II were pooled, lyophilized, dissolved in Tris-HCl-urea buffer, and applied to a DEAE-cellulose column. When the column was eluted with 0.01 M NaCl, a small amount of immunoreactivity (DEAE-IIa) appeared. When eluted with 0.1 M NaCl, a second peak (DEAE-IIb) containing more than 90% of the immunoreactivity was obtained (Fig. 3). Fractions 30 to 33 corresponding to the peak of DEAE-IIb were pooled, dialyzed against distilled water, lyophilized, and stored at -20° for subsequent gel-electrophoresis and gel-filtration analyses.

Polyacrylamide-gel-electrophoresis and gel-filtration analysis of glucagon immunoreactive protein purified on DEAE-cellulose. The homogeneity of DEAE-IIb was assessed by polyacrylamide disc gel electrophoresis in urea-containing gels, at pH 3.5, and a single amido black-stained band was obtained (Fig. 4). Because of scarcity of

material, we estimated the molecular size of the purified protein by labeling it with ^{125}I (19) and chromatographing it on a Sephadex G-50 column, eluted with 1 M acetic acid. Figure 5 shows a major radioactive peak of approximately 9000 daltons. Minor components, appearing at the void and the salt volume, probably represent degraded labeled peptides adsorbed to serum albumin and free iodide, respectively.

Amino acid analysis of DEAE-IIb protein. The amino acid analysis of the DEAE-IIb fraction obtained from the islets of 3 birds revealed the presence of 76 residues.

Discussion. Using inhibitors of proteolysis in the extraction procedure, we have partially purified a "large glucagon" molecule from pigeon pancreatic islets. The protein purified on DEAE-cellulose appeared homogeneous by polyacrylamide-gel electrophoresis, reacts with anti-glucagon sera, and has a size of approximately 9000 daltons. Amino acid analysis showed that this protein resembles angler fish "proglucagon" (20) and contains all the residues of turbot glucagon (21). The material appears to be susceptible to proteolysis, as indicated by

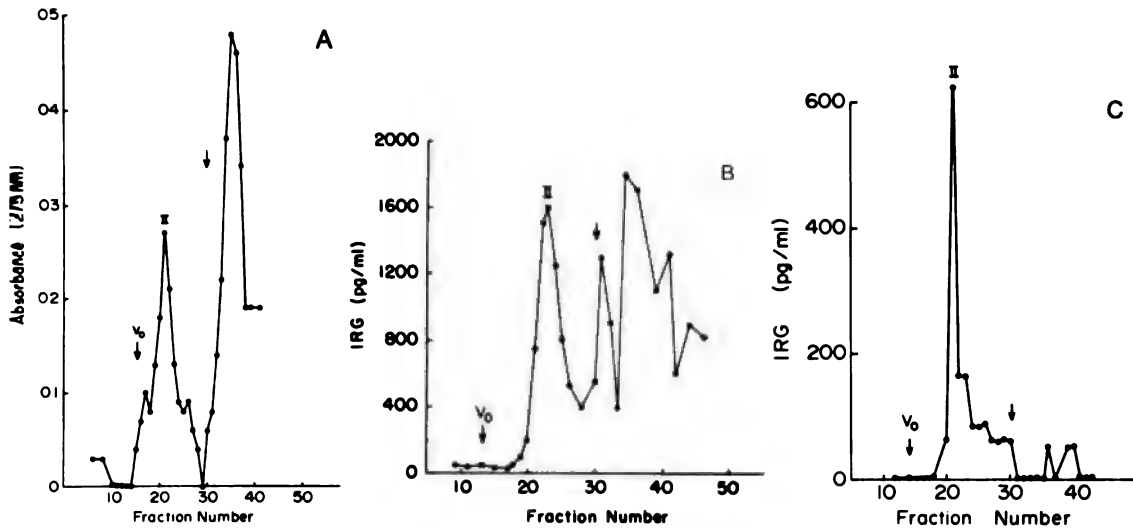


FIG. 2. (A) Rechromatography of Peak II (Fig. 1) on a Sephadex G-50 column (60 \times 0.9 cm). Fraction volume, 1 ml. Peak II was obtained as described in the legend of Fig. 1. Results correspond to experiments in which islets were processed without inhibitors of proteolysis. (B) An aliquot of 0.1 ml from each fraction was lyophilized with 0.01 ml of aprotinin solution (1000 kiu). Lyophilized fractions were dissolved in 0.7 ml of sodium barbital-albumin buffer for glucagon radioimmunoassay. (C) Rechromatography of Peak II on a Sephadex G-50 column. Results correspond to an experiment in which islets were processed in the presence of aprotinin.

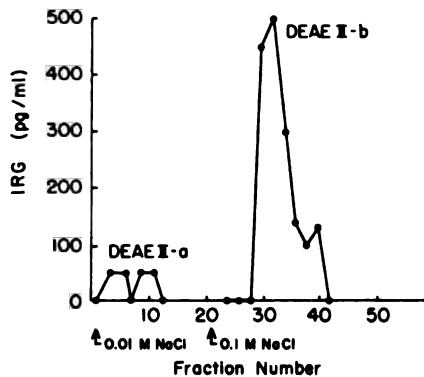


FIG. 3. DEAE-cellulose ion-exchange chromatography of Peak II (Fig. 2) after refractionation on a Sephadex G-50 column. Proteins, extracted from islets in the presence of benzamidine-HCl, were chromatographed. Aliquots of 0.1 ml in 0.1 ml of aprotinin solution (1000 kiu) were dialyzed against distilled water. The material was lyophilized and dissolved in 0.5 ml of sodium barbital-albumin buffer for radioimmunoassay.

degradation into glucagon and smaller immunoreactive forms during fractionation of the crude extract by gel filtration. These data are consistent with the hypothesis that Peak II contains the glucagon molecule in its



FIG. 4. Polyacrylamide gel-electrophoresis of DEAE-Peak-IIb. Fractions corresponding to DEAE-Peak-IIb (Fig. 3) were pooled and dialyzed against distilled water. The protein recovered by lyophilization was dissolved in 100 μ l of 0.01 M HCl and subjected to electrophoresis in urea-containing gels, pH 3.5 (12). (1) Pancreatic glucagon; (2) DEAE-Peak-IIa; (3) DEAE-Peak-IIb. +, Anode; -, cathode.

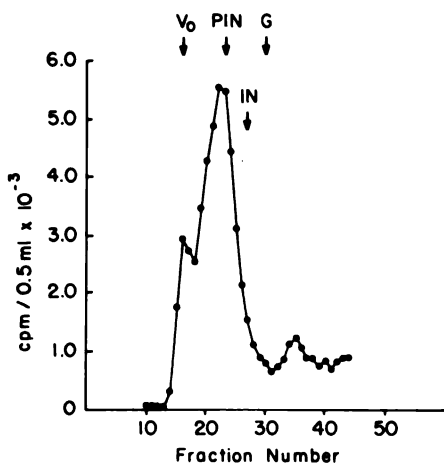


Fig. 5. Sephadex G-50 gel filtration of labeled DEAE-Peak-IIb. Column size, 60×0.9 cm. Fraction volume, 1 ml. The protein was iodinated with carrier-free [125 I]iodide by the chloramine-T method of Greenwood and Hunter (19). PIN, elution volume of bovine proinsulin (gift of Dr. C. Yip, University of Toronto, Canada); IN, insulin; G, glucagon.

primary structure and that it is not a glucagon polymer.

Recent studies on the biosynthesis of glucagon suggest the existence of biosynthetic precursors or intermediates having molecular weights of 69,000 (5), 18,000 (7), 9000, 6000 (6-8), and 4500 daltons (22). It is not known if these substances are identical to the large glucagon immunoreactive materials found in the crude extracts of pancreas and isolated islets (9, 10); however, it has been suggested that the biosynthesis of pigeon glucagon may proceed through a 9000-dalton intermediate (6). In these studies, labeled Peak II obtained from islets incubated with 3 H-labeled tryptophan coeluted with a glucagon immunoreactive fraction of 9000 daltons on a Sephadex G-50 column. Polyacrylamide-gel electrophoresis of the same material showed two radioactive peaks which coincided with two protein bands associated with glucagon immunoreactivity. However, gel-filtration data also revealed a prominent immunoreactive peak of about 6000 daltons, eluting after the labeled Peak II, and electrophoretic data showed an immunoreactive component more cationic than the labeled peak (6). In the present study, using an improved extraction procedure, the purification of Peak II by gel filtra-

tion yielded only the 9000-dalton immunoreactive component.

Although these results suggest that the 9000-dalton glucagon immunoreactive protein may be a biosynthetic component, final characterization of this protein as "proglucagon" must await determination of its amino acid sequence.

Summary. Pigeon pancreatic islets were homogenized in acid ethanol containing inhibitors of proteolysis. Purification of the extract, including fractionation on Sephadex G-50 columns, yielded a 9000-dalton protein, which after further purification by DEAE-cellulose chromatography, appeared to be homogeneous and more cationic than porcine glucagon on polyacrylamide disc gel electrophoresis. When the protein obtained by DEAE-cellulose chromatography was iodinated and applied to a Sephadex G-50 column, a single radioactive peak of 9000 daltons was also obtained. The DEAE-cellulose peak is composed of 76 amino acids, including all those of turkey glucagon. Its amino acid composition is similar but not identical to that of angler fish "proglucagon".

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Acid Excretion in Spontaneously Hypertensive Rats¹ (39543)

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Spontaneously hypertensive rats (SHR) (1) offer a unique opportunity to study the pathogenesis of genetic hypertension in an animal model. Since it has been proposed that human essential hypertension may be related to altered renal function, it occurred to us that genetic hypertension might be associated with renal malfunction as well (2, 3). Recent evidence suggests that kidneys are involved, at least in part, in the pathogenesis of the genetic hypertension in rats (4). Our initial interests have been to compare renal function in SHR with normotensive Wistar rats (NWR). Toward this end, we have examined PAH transport and oxygen consumption in kidney slices from SHR and NWR and found differences in these functions (5). The purpose of the present investigation was to extend our knowledge of renal function in SHR by assessing acid excretion. We found that, compared to two normotensive strains of rats, SHR showed decreased acid excretion secondary to depressed ammonium excretion.

Methods. Experiments were initiated on 11 normotensive male Sprague-Dawley rats with an average weight of $237 \text{ g} \pm 15.0$ (SEM) (Flow Laboratories, Dublin, Va.), on 50 normotensive male Wistar rats with an average weight of $455 \text{ g} \pm 19.6$ (SEM) (Microbiological Associates, Walkersville, Md.), and on 56 spontaneously hypertensive male Wistar rats with an average weight of $347 \text{ g} \pm 9.2$ (SEM) (Taconic Laboratories, Germantown, N.Y.). All rats were housed in a constant-temperature room with a light-dark phase of 14 and 10 hr, respectively, and given Purina rat chow and water *ad libitum*. To study acid excretion under normal acid-base conditions, rats were

given 2 ml/100 g BW of water by stomach tube, and urine was collected over the ensuing 4 hr. In other studies, rats were made acutely acidotic by giving them 1 mmole/100 g BW of 0.5 M ammonium chloride solution administered by stomach tube. After this, the rats were placed in metabolic cages and timed 4-hr collections of urine were obtained. To produce chronic acidosis, rats received 1 mmole/100 g BW of 0.5 M ammonium chloride b.i.d. by stomach tube. On the third day, urine was collected over the 4 hr following the morning dose of NH_4Cl .

Some of the rats were anesthetized lightly with ether, blood was drawn from the aorta, and the kidneys were removed and immediately placed in cold saline either following the collection of urine after water load or after the urine collection following chronic acid challenge. The decapsulated kidneys were weighed to the nearest milligram.

Using a Radiometer pH meter equipped with an automatic titrator, we estimated pH and determined titratable acidity by titrating the urine to pH 7.4 and 0.01 M NaOH. Ammonia was measured by the method of Preuss *et al.* (6). Carbon dioxide content was determined and calculated as described by Natelson (7). No blood pH's were determined; and so, for our purposes, we assumed that CO_2 content in millimoles per liter = millequivalents of HCO_3^- per liter (8). Blood pressure (BP) was estimated by tail plethysmography (9). At least three readings of BP from different days were taken for each rat.

Kidney slices, cut to approximately 0.4-mm thickness with a Stadie-Riggs microtome (10), were trimmed so as to weigh 50 mg and were placed in 5 ml of incubation medium containing NaCl, 110 mM; KCl, 5 mM; MgSO_4 , 1.2 mM; CaCl_2 , 1 mM; NaHCO_3 , 25 mM; and NaH_2PO_4 , 1.2 mM. Glutamine (2 mM) was added to the me-

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ium in some studies. The medium was assed with 95% O₂ and 5% CO₂ at 37°. initial pH was 7.4 and after incubation was elow 7.5. After an incubation of 90 min, media from the flasks were deproteinized ith cold 1.2 N perchloric acid. Following entrifugation, the perchloric acid was pre- ipitated by the addition of KOH, phos- hate buffer mixture (final pH 7.0), and mmonia determinations were made on the ipernates.

Statistics were performed by Student's *t* test with statistical significance set at *P* < .05.

Results. In Table I are summarized results f acid excretion in normotensive Sprague-Dawley rats (SD), BP < 125 mm Hg; nor- otensive Wistar rats (NWR), BP < 125 mm Hg; and hypertensive Wistar rats (SHR), BP > 150 mm Hg. Following wa- r loading, the pH of the SHR was higher an in either normotensive species of rat. In addition, ammonium excretion was less. After acute acid challenge, the volume ex- etion of SHR was lower than the NWR at not lower than the normotensive SD. While the urine pH and titratable acid were o different among the groups, ammonium cretion was significantly lower in the HR. Following chronic acid challenge, the ine volume excretion was lower in the HR than the other two groups, the urine

pH's were similar, the titratable acids were higher in SD than the other two groups, and, again, ammonium excretion was lower in the SHR. Serum HCO₃⁻ concentrations (CO₂ content) were estimated in some of the rats. The serum HCO₃⁻ in nine SHR on the third day of acidosis (15.2 μ equiv/liter \pm 1.2 (SEM)) was lower than that in nine normotensive Wistar rats (18.7 μ equiv/liter \pm 1.2 (SEM)). These differences just miss statistical significance, 0.05 < *P* < 0.1.

Figure 1 depicts the relationship between kidney size and body weight in 50 normo- tensve Wistar rats and 56 spontaneously hypertensive Wistar rats. No obvious differ- ence between the two groups is noticeable.

Figure 2 depicts results obtained by study- ing ammoniagenesis in kidney slices from SHR and NWR. Ammonia production was not different between slices from either group whether no substrate was present in the medium (endogenous production) or in the presence of 2 mM glutamine. However, when slices were removed from rats on the third day of acidosis, SHR slices showed less "endogenous" and "glutamine" ammoni- agenesis.

Discussion. Following acute acid chal- lenge, normal kidneys excrete more H⁺ as titratable acid and ammonium (11). If the challenge continues, urinary ammonium in- creases more and accounts for the majority

TABLE I. ACID EXCRETION IN NORMOTENSIVE AND HYPERTENSIVE RATS.^a

Rat	Number	Urine volume (ml/4 hr)	Urine pH	Titratable acid (μ equiv/hr/100 g BW)	Ammonium (μ equiv/hr/100 g BW)
Water load					
SD	11	4.8 \pm 0.6	6.9 \pm 0.2		9.4 \pm 1.7
NWR	22	8.5 \pm 0.4	7.2 \pm 0.1		5.8 \pm 0.6
SHR	25	6.2 \pm 0.3 ^b	7.8 \pm 0.1 ^c		4.6 \pm 0.6 ^c
Acute acid challenge					
SD	7	6.3 \pm 1.1	5.9 \pm 0.9	6.6 \pm 2.0	38.7 \pm 5.1
NWR	30	9.9 \pm 0.4	6.0 \pm 0.1	7.8 \pm 1.3	39.6 \pm 1.4
SHR	29	5.8 \pm 0.5 ^b	5.9 \pm 0.1	6.8 \pm 0.8	28.5 \pm 1.6 ^c
Chronic acid challenge					
SD	7	6.4 \pm 0.7	5.7 \pm 0.6	11.7 \pm 1.3	70.2 \pm 8.3
NWR	26	6.7 \pm 0.4	5.9 \pm 0.1	6.8 \pm 0.1	72.4 \pm 3.8
SHR	25	4.6 \pm 0.4 ^c	5.9 \pm 0.1	5.6 \pm 0.6 ^d	54.9 \pm 4.1 ^c

^a SD = normotensive Sprague-Dawley rats, NWR = normotensive Wistar rats, SHR = spontaneously hypertensive Wistar rats. Values are means \pm SEM.

^b *P* < 0.05 compared to NWR.

^c *P* < 0.05 compared to SD and NWR.

^d *P* < 0.05 compared to SD.

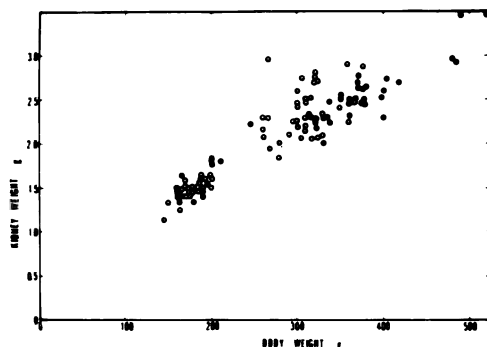


FIG. 1. Correlation between kidney weight (KW) and body weight (BW) in normotensive Wistar rats (NWR) (●) and spontaneously hypertensive Wistar rats (SHR) (○).

of H^+ excreted (11). Van Slyke *et al.* first reported that the source of renal ammonia is the amide nitrogen of glutamine (12). Subsequently, other studies verified this and added that the amino nitrogen of glutamine also contributes to the formed ammonia (13–15), and that the remaining carbon skeleton becomes a major renal fuel during acidosis (16, 17).

In the present study, we assessed the response of SHR to acid challenge. Compared to normotensive Wistar and Sprague-Dawley rats, hypertensive Wistar rats showed a similar ability to lower urine pH and excrete titratable acid but less ability to increase ammonium excretion following acute or chronic acid challenge. The extent of acid challenge among the groups appeared to be similar. When serum HCO_3^- concentrations were checked in a few chronically acidotic rats, systemic acidosis, if anything, was more severe in SHR than NWR.

Why the decrease in ammonium excretion in SHR? Ammonium excretion can be affected, for the most part, by three factors: urine volume, urine pH, and renal ammonia production. The first two are factors because ammonia (NH_3) is excreted into the tubular lumen by nonionic diffusion as a gas (where it reacts with H^+) to form ammonium (NH_4^+) (18, 19). Both increased urine flow and decreased urine pH lower urine pNH_3 and allow more diffusion of free base NH_3 from the renal cells and the peritubular blood stream. Richterich (20) has performed extensive studies relating urine flow

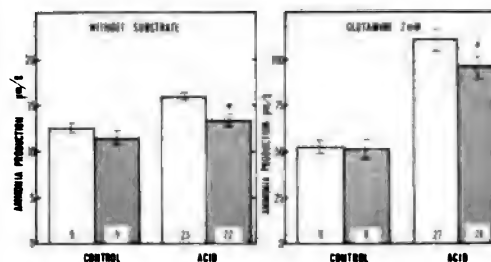


FIG. 2. Histograms depicting ammoniogenesis by kidney slices from normal (control) and acidotic normotensive Wistar rats □ and spontaneously hypertensive Wistar rats ■. Slices were incubated without substrate or in the presence of 2 mM glutamine. The scales for these two situations differ. Numbers at the base of the columns indicate the number of rats studied. (*) $P < 0.05$. SEMs are shown by the vertical lines.

to urine ammonium excretion in humans and dogs. At higher urine pH, flow can enhance excretion but becomes less of a factor at lower pH's, e.g., below pH 6.5 (21). In our studies, there was no consistent correlation between urine flow and ammonium excretion at high or low urine pH's. Sprague-Dawley rats, after water loads, put out a lower urine volume and still excreted more ammonium. During acute acid challenge, the differences in urine flow between Sprague-Dawley rats and SHR were not significantly different. Because we could find no consistent relationship between urine flow and ammonium excretion, we believe that changes in urine flow could not be the only factor in bringing about differences in ammonium excretion here. In addition, urine pH could not be a major factor; for during acute and chronic acid challenge, urine pH's were similar.

The above reasoning suggests that lesser renal ammonia production is the basis for the lowered urinary excretion of ammonium. Could kidney size, i.e., the amount of ammonia producing mass, play a role (22)? No. If anything, the younger Sprague-Dawley rats were smaller rats when studied and had smaller kidneys. Additionally, although NWR are larger than SHR of the same age (23), Fig. 1 shows that the kidney weight to body weight ratio was similar in the two groups of Wistar rats. It follows that, when we report ammonium excretion per 100 g BW (see Table I), we are, in essence, taking

ze into consideration. Therefore, the differences between groups are possible for the differences in ammonium excretion (Table I).

We have no definitive answer as to how ammoniogenesis is different in our *in vitro* studies suggest that this can be attributed to intrinsic metabolic differences. Ammonia produced by kidney slices from NWR and normal acid-base balance was not in the presence or absence of glutamine. This seems strange as slices from SHR have a higher oxygen consumption and this is usually associated with a capacity to produce ammonia in normal SD (24). In addition, slices from chronically acidotic SHR compared to slices from chronically acidotic NWR produce less ammonia in either medium. This 12% decrease in production is considerably less than the decrease in ammonium excretion in SHR compared to NWR following chronic acidosis (−24%). Therefore, differences in intrinsic renal metabolism during acidosis may be partially responsible for the differences in excretion but probably do not explain the entire difference.

We believe that additional factors are affecting ammonia production *in vivo*. Circulatory factors affect the metabolism of glutamine in renal cells (25, 26). Any changes in substrate concentrations (27) or stimulation (28) to ammonia production could result in changes that would not necessarily be mirrored in slice studies. In addition, if acidosis markedly affects renal blood flow or distribution, this might result in glutamine being presented to ammonifying cells in kidneys. This is a possibility that should be checked out. We know that the vascular system of SHR tends to be hyperreactive (29).

Does this finding relate directly or indirectly to the pathogenesis of hypertension—whether this defect is present in animals with essential hypertension is uncertain. That SHR and patients with essential hypertension both have an exaggerated acid response is known (30), but we are lacking any conclusive studies on ammoniogenesis in patients with essential hypertension.

Summary. SHR excrete less ammonium than NWR and SD following water load and acute and chronic acidosis. Thus, their ability to handle acute and chronic acid challenge is impaired because of the lesser ammonium excretion. Since these differences in ammonium excretion cannot be related in entirety to urine flow, urine pH, and kidney mass, basic differences *in vivo* in renal metabolism of glutamine, the major precursor of renal ammonia, seem likely. Slices from chronically acidotic SHR compared to slices from chronically acidotic NWR produce less ammonia, suggesting some basic intrinsic difference in renal glutamine metabolism during persistent acidosis. Because these differences in slice ammoniogenesis are smaller than the differences in ammonium excretion, and because slices from nonacidotic SHR and NWR show no differences in ammoniogenesis, we feel that other *in vivo* factors, probably extrarenal, additionally decrease renal ammonia production in SHR.

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Experimental Hepatomas: Dehydrogenation of Reduced Pyridine Nucleotides by the D-T Diaphorase¹ (39544)

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In this communication, the enzymatic activity of the soluble D-T diaphorase (reduced NAD(P): (acceptor) oxidoreductase (EC 1.6.99.2)) (1) in experimental hepatomas is reported. These studies were prompted by the fact that Leydig cell tumors of the rat testis show a D-T diaphorase activity several times that of non-neoplastic interstitial tissue (2). This finding has led to the hypothesis that the increased enzyme activity represents an anaplerotic mechanism compensating for the disappearance from neoplastic tissues of the shuttles normally used in the oxidation of extramitochondrial NADH and NADPH (3).

The dehydrogenation of NADH and NADPH by mitochondrial and microsomal enzymes has been studied in experimental hepatomas. It has been found that the NADH- and NADPH-cytochrome *c* reductases are decreased in the mitochondria and microsomes of the more undifferentiated hepatomas (4-6). Furthermore, the activity of the microsomal mixed oxidases are either reduced or absent in microsomes obtained from hepatomas (6, 7).

The slowly growing 7787 hepatoma and the rapidly growing 5123tc hepatoma were used in this study.

Materials and methods. The animals were killed by sudden decapitation. The livers of the control animals, the livers of the host rats, and the hepatomas were immediately dissected and homogenized. A 10% homogenate in 0.25 *M* sucrose buffered with Tris, pH 7.4, 0.1 *M* (the same results were obtained when potassium chloride 0.154 *M* with phosphate buffer, pH 7.4, 0.1 *M*, was used in the centrifugation medium), was

prepared from the different specimens and then subjected to differential centrifugation in a Spinco L preparative ultracentrifuge. The supernatant was obtained after the centrifugation of the postmitochondrial fraction for 2 hr at 105,000g.

The enzymes were assayed in a Unicam recording spectrophotometer using dichloroindophenol (DCPIP) as an acceptor and NADH and NADPH as substrates. The composition of the incubation medium was phosphate buffer, 3.3×10^{-2} *M*; DCPIP, 3.33×10^{-5} *M*; and NADPH and NADH, 7.6×10^{-5} *M*. The final volume was 1.3 ml. In each determination approximately 70 μ g of protein were used. The reaction was started with either NADH or NADPH, and the reduction of DCPIP was followed for 1 min at 600 nm. The extinction coefficient of 22.1 $\text{mM}^{-1} \text{cm}^{-1}$ was used for the determination of the enzyme activity. Protein was determined by the method of Nayyar and Glick (8).

Results. The results obtained with the 7787 tumor are shown in Table I. Experiments with the 5123tc tumor are shown in Table II. The enzymatic activity in the experimental hepatomas is more than four times that of normal liver and three times that of the host liver. Also, from these results it can be seen that the increased activity of the D-T diaphorase is maintained throughout the different generations of the 5123tc tumor. It can also be observed that the host livers exhibit a higher activity than the livers of the control animals. These findings will be discussed elsewhere.

The kinetics of DCPIP reduction by NADH and NADPH in the tumors has been studied. The apparent Michaelis constant appears, with small variations, to be the same in the liver of the control and of the host animals and the hepatomas. Although

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the concentration of NADH and NADPH used in these experiments was near the K_m concentration, similar differences in activities were observed when the substrate concentration was either lowered fivefold or increased five- and tenfold, respectively. These results will be communicated in full in a forthcoming paper.

A critique often made of this type of work is that the enzymatic activity observed in the supernatant may constitute an artifact produced by a leakage of particulate enzymes into the cytoplasm. When the activities of the cytochrome *c* reductases of the particulate fractions of livers and hepatomas were compared with those of the supernatants, the degree of reduction of cytochrome *c* was

tenfold higher using NADH as a substrate (the same results are obtained if DCPIP is used as an acceptor instead of cytochrome *c*); whereas in the supernatant the degree of reduction of DCPIP was almost equal using either NADPH or NADH as the substrate. Furthermore, the soluble enzymes will reduce cytochrome *c* only if a mediator such as menadione is present in the incubation mixture; meanwhile, the particulate enzymes reduce DCPIP without an electron mediator. These determinations tend to indicate different properties of the particulate and soluble enzymes which dehydrogenate reduced pyridine nucleotides in normal livers; these differences are retained in hepatomas. Thus, it appears that a real increase of activity of the D-T diaphorase is observed in experimental hepatomas and that this increase is not an artifactual result of the procedures used. The enzyme was inhibited by Dicumarol, hydroxycoumarin, and 7,8-benzoflavone. When other hydrogen acceptors such as cytochrome *c* and nitroblue tetrazolium plus vitamin K_3 were used, the same differences between normal livers and hepatomas were observed.

Discussion. The interpretation of these findings at this moment is speculative. A possible interpretation is that this increase in activity is an anaplerotic mechanism to substitute for the decreased activity of the

TABLE I. D-T DIAPHORASE ACTIVITY HEPATOMA 7787.^a

Substrate	NADH	NADPH
Control liver	387.7 ± 31.6 (6)	468.1 ± 36.2 (6)
Hepatoma (<i>t</i> :23, <i>d</i> :65) ^b	1462.7 ± 151.2 (6)	2021.4 ± 265.2 (6)
Host liver	434.4 ± 86.5 (6)	454.7 (6) ± 122.2

^a Activities expressed as micromicromoles of reduced DCPIP per microgram of protein present in the incubation mixture per minute in the soluble fraction obtained after centrifugation at 105,000g/2 hr.

^b *t*, Number of generations of the tumor; and *d*, days of inoculation of the tumor before sacrifice.

TABLE II. D-T DIAPHORASE ACTIVITY HEPATOMA 5123tc.^a

Substrate	NADH	NADPH
Experiment 1		
Control liver	210.7 ± 20.5 (5)	284.2 ± 44.1 (5)
Hepatoma (<i>t</i> :128, <i>d</i> :25) ^b	1153 ± 162.2 (6)	1374.1 ± 246.1 (6)
Host liver	320.8 ± 50.64 (6)	367.50 ± 50.1 (6)
Experiment 2		
Control liver	149.70 ± 20.3 (8)	219.2 ± 28.5
Hepatoma (<i>t</i> :135, <i>d</i> :26) ^b	878.3 ± 99 (7)	1270.4 ± 195.1 (7)
Host liver	201 ± 29.2 (8)	306 ± 30.1 (8)
Experiment 3		
Control liver	254.3 ± 48.8 (6)	276.6 ± 54.9 (6)
Hepatoma (<i>t</i> :138, <i>d</i> :45) ^b	1186.42 ± 127.7 (6)	1474 ± 257.7 (6)
Host liver	419.14 ± 77.4 (6)	514.1 ± 57.8 (6)

^a Activities expressed as micromicromoles of reduced DCPIP per microgram of protein present in the incubation mixture per minute in the soluble fraction obtained after centrifugation at 105,000g/2 hr. In parentheses, the number of animals used.

^b *t*, Number of generations of the tumor; and *d*, days of inoculation of the tumor before sacrifice.

shuttles used in the oxidation of NADH and NADPH by neoplastic mitochondria (3). It is known that the endogenous O_2 consumption of hepatoma mitochondria is enhanced (9); therefore, the D-T diaphorase could be the enzyme responsible for the transfer of reducing equivalents to the mitochondria. This explanation agrees with observations made by Conover and Ernster on the function of this enzyme (10, 11). These workers have postulated that the enzyme can transfer reducing equivalents to the mitochondrial chain using vitamin K_3 as an intermediate. In ascites tumor cells, it has been shown that glucose can contribute reducing equivalents for respiration only if vitamin K_3 is present (12). Although a diaphorase-type activity was suggested to explain these findings, the specific activity of the enzyme was not measured. A recent report by Lanoue *et al.* (13) on the defects of anion and electron transport in Morris hepatoma mitochondria has shown that the iron-sulfur protein which transfers electrons from NADH to ubiquinone in mitochondria is decreased in neoplasia. These authors have also confirmed and enlarged previous observations of Boxer and Devlin (3) on the activity of the shuttles utilized in the transfer between reduced pyridine nucleotides and mitochondria.

The extent of the defect in mitochondrial respiration according to Lanoue and her collaborators (13) does not appear to be a function of growth rate. Likewise, the results reported in this communication regarding the activity of the D-T diaphorase in the rapidly growing 5123tc and slowly growing 7787 hepatomas does not correlate with the growth rate of these hepatomas.

A second alternative may be deduced from a recent report which has shown that the D-T diaphorase may be related to the aryl hydrocarbon hydroxylase (AHH) system of liver microsomes (14). Microsomal mixed oxidases are greatly reduced or absent in microsomes obtained from hepatomas (6, 7). Although the relation of the D-T diaphorase to the hydroxylative system is not yet clear, it has been shown that the activity of the enzyme can be induced *in vivo* by methyl cholanthrene which induces the AHH system of liver microsomes (13).

Likewise, 7,8-benzoflavone (12), an *in vitro* inhibitor of the AHH system, suppresses the activity of the D-T diaphorase extracted from either liver or hepatomas. Therefore, it may be postulated that the increase of the activity of the enzyme may be related to this process. Experiments conducted in our laboratory have shown that the activity of the enzyme is increased three- and twofold when rats are fed dimethylbenzanthracene and acetylaminofluorene. These changes are observed 24 hr after the administration of the carcinogen (15). The reducing equivalents transferred by the enzyme may also be utilized for the conversion of ribonucleotides to deoxyribonucleotides. This biochemical function is increased in hepatomas and is localized in the postmicrosomal fraction (16).

Summary. Major differences were observed in the dehydrogenation of reduced pyridine nucleotides when experimental transplantable hepatomas were compared with livers of control animals and livers of tumor-bearing animals. The enzymatic activity of the D-T diaphorase was increased almost fivefold in the hepatomas. The same increase in enzymatic activity was observed in one slowly growing and one rapidly growing Morris hepatoma. The possible functional significance of these findings is discussed.

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Postnatal Changes of Water and Electrolytes of Rat Tissues (39545)

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Electrolyte and water contents of neonatal rat tissues are different than those of mature animals. Cellular and extracellular water content is high, sodium content is relatively high, and potassium content is low (1). Detailed examination of water and electrolyte changes during development for other than muscle (1, 3) and brain (2) has not been done. The purpose of this study was to determine whether water and electrolyte regulation mature at the same time during postnatal development in several tissues: heart, liver, kidney, spleen, and testis.

Methods. Young rats (Simonsen-Wistar, Calif.) were selected from litters born in our animal quarters. Two males and two females were used at each time point studied. The only exception to this was that only one male and two females were analyzed at 60 days and only one of each sex at 100 days. The number of animals remaining in each litter was approximately the same in order to insure that litter sizes were reasonably matched.

Animals were anesthetized with ether. The thorax was opened, the heart was removed, and the ventricles were separated, weighed, and weighed on aluminum tares. The abdomen was then opened and the following organs were removed in sequence: lungs, stomach, and extraneous tissue; blotted, and weighed on individual tares; liver, spleen, and ovaries (from animals over 23 days of age). Kidneys were removed and weighed. Both kidneys and testes of individual rats were pooled for analysis in 9-day-old rats. In animals 16 days of age or older, kidney cortex was separated from medulla. No analyses were carried out on tissues of animals younger than 16 days. Tissues were weighed and then dried at constant weight for determination of water content. The tissues were placed in a desiccator containing 10 ml of distilled water and dried at 4° for at least 2 weeks. Sodium

and potassium contents were then determined using an IL emission spectrophotometer.

Although there are differences between body and organ weights of male and female rats at older ages, no consistent and reproducible differences in electrolyte contents or in water contents were found between tissues of animals of the same age; therefore, data from both sexes are pooled for the remaining tissues except ovary and testis. All data are expressed as means plus or minus standard errors. Regression analysis was carried out using the method of least squares and differences are considered to have statistical significance when $p < 0.05$.

Results. In Fig. 1, water contents are shown as a logarithmic function of age. During the postnatal period, the tissue containing the least amount of water is liver, followed by heart, kidney, and spleen having similar water contents. The sex organs have the highest water content with the water content of the testis exceeding that of the ovary.

The most striking feature with respect to water content is that, in liver, heart, kidney, and spleen, the changes undergo a biphasic pattern which is evident from the semilogarithmic relationship shown in Fig. 1. In these tissues, there is a phase of rapid decline in water content ending at 23 days, followed by a phase of much slower decline during which changes in water content are much less and where absolute values approximate mature values. The testis does not show any significant age-dependent change. The ovary shows a gradual decrease in water without any clear inflection.

Sodium and potassium contents of the tissues are shown in Table I. The ratio of Na/K as a function of age is shown in Fig. 2. Changes in Na/K ratios do not parallel the changes in water content. As might be expected from high extracellular fluid (ECF), tissue sodium content is high relative to po-

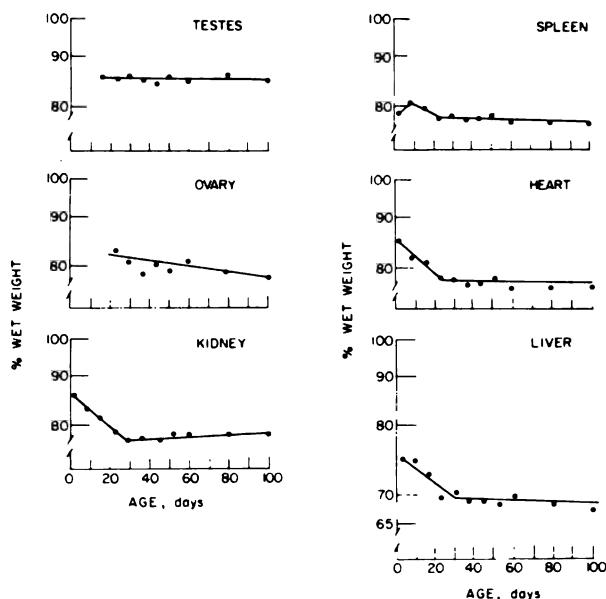
POSTNATAL NA, K, AND H₂O CONTENTS

FIG. 1. Water content as a percentage of wet weight for the various tissues studied. Each panel shows data for a single organ.

tassium in the early postnatal period and undergoes a decrease (Table I and Fig. 2). In contrast to water content, however, the Na/K ratio reaches a minimum when the rats are 37 to 44 days old; thereafter, a rise in Na/K ratios occurs for liver, spleen, kidney cortex, and testis, while heart maintains a constant Na/K ratio.

Electrolyte contents of papilla and ovary (Table I) show different patterns. Papillary sodium content is low at 16 days of age. Sodium content is much higher thereafter as might be expected from the observation that the concentrating mechanism assumes mature characteristics during the third week of life in rats (1, 5). An unexpected finding is that ovarian sodium content was found to parallel the papillary pattern over those age ranges where we could measure ovarian electrolytes. In Fig. 3, average ovarian Na is plotted as a function of the average Na in the papilla for each female rat in this study. It is evident that the values of both ovary and papilla increase in parallel. These values have been fitted by a linear least-squares relationship, the equation being $Na_{ov} = 132 + 0.679 Na_{pap}$. The r value of 0.606 ($p < 0.02$) shows that there is a significant correlation.

In Fig. 4, the sum of Na and K (expressed

per kilogram of tissue water) is shown as a function of age for each tissue except ovary and papilla. When examined in this way, a maximum in cation content occurs at 16 days of age for heart, kidney, and spleen. At later times, there is a continuous sharp decrease in the sum of Na and K until about 40 days. A gradual decrease may continue thereafter. In the case of liver, the changes are insignificant.

Discussion. The results of this study demonstrate a dissociation between the decrease in water content and the changes in Na/K ratio for all of the tissues analyzed except for papilla and ovary, similar to that found by others in muscle and brain (5, 6). It is of interest that the reduction in water content occurs at the time of weaning, which is also a "critical time" for the development of urinary-concentrating ability (5, 7) and spontaneous reduction of plasma renin activity (8). On the other hand, the Na/K ratio reaches its minimal value at 35–45 days of age. At this time, the kidney of the rat becomes able to respond to blood volume expansion (9), and baseline excretion of sodium and potassium reach mature levels (10). It would appear that in the postnatal period, one finds a dissociation of development of water and electrolyte regulation. One can also suggest

TABLE 1. ELECTROLYTE CONTENTS AS A FUNCTION OF AGE.*

Age (days)	Heart		Liver		Ovary		Spleen		Kidney cortex		Papilla		Testis	
	Na	K	Na	K	Na	K	Na	K	Na	K	Na	K	Na	K
2	—	—	38.6 ± 3.2	84.5 ± 6.3	—	—	—	—	85.4 ± 6.7*	29.6 ± 7.7*	—	—	—	—
9	82.7 ± 15.5	64.3 ± 15.7	23.1 ± 1.3	75.7 ± 1.9	—	—	51.6 ± 12.7	112 ± 6.2	57.8 ± 4.4*	73.0 ± 1.9*	—	—	—	—
16	71.7 ± 9.8	81.5 ± 2.7	29.0 ± 8.1	71.6 ± 5.5	177	31.3	121 ± 16.2	114 ± 3.8	114 ± 5.5	76.0 ± 3.2	76 ± 19.7	23.3 ± 0.45	11 ± 0.65	—
23	71.2 ± 3.6	92.7 ± 2.0	20.0 ± 2.7	76.9 ± 0.9	526	305	56.6 ± 5.0	118 ± 11.6	75.3 ± 3.3	85.9 ± 2.1	426 ± 45.9	105 ± 11.0	109 ± 1.0	—
30	53.3 ± 2.0	81.3 ± 3.1	18.3 ± 1.1	78.7 ± 2.7	455	76.1	36.0 ± 1.9	111 ± 3.5	57.7 ± 1.6	75.4 ± 1.8	347 ± 14.3	55.8 ± 2.0	95.3 ± 0.24	—
37	36.6 ± 1.8	81.6 ± 2.0	14.6 ± 3.3	79.7 ± 1.3	179 ± 30.2	169 ± 33.7	22.3 ± 0.34	122 ± 1.9	41.3 ± 8.8	72.2 ± 1.8	160 ± 6.5	38.8 ± 2.4	85.8 ± 1.6	—
44	36.1 ± 4.9	79.2 ± 2.3	15.8 ± 2.9	76.9 ± 1.5	304 ± 127	—	24.0 ± 1.7	131 ± 7.7	49.5 ± 7.5	77.8 ± 2.1	393 ± 48.3	39.8 ± 1.6	92.6 ± 1.9	—
51	39.2 ± 0.75	82.6 ± 4.4	15.0 ± 1.4	71.4 ± 4.5	252	43.2	23.9 ± 1.6	127 ± 5.9	46.4 ± 3.2	70.3 ± 1.5	238 ± 74.5	38.8 ± 1.6	88.7 ± 4.8	—
60	32.8 ± 1.0	75.1 ± 1.0	17.7 ± 1.2	78.9 ± 1.9	156 ± 15.6	74 ± 1.1	24.4 ± 0.41	111 ± 2.3	43.8 ± 1.5	60.5 ± 3.4	169 ± 6.4	35.6 ± 0.88	75 ± 0.83	—
80	30.3 ± 1.1	75.6 ± 6.1	15.2 ± 1.1	68.4 ± 9.7	301 ± 47.3	162 ± 13.3	19.5 ± 2.2	102 ± 11.6	40.3 ± 2.0	72.7 ± 4.5	177 ± 26.1	32.8 ± 1.5	90.1 ± 6.9	—
100	35.8	74.5	16.6	74.7	363	142	36.6	107	45.6	63.7	284	46.7	80.9	—

* All data are reported as milliequivalents per kilogram of wet weight.

* These data represent whole kidney electrolytes.

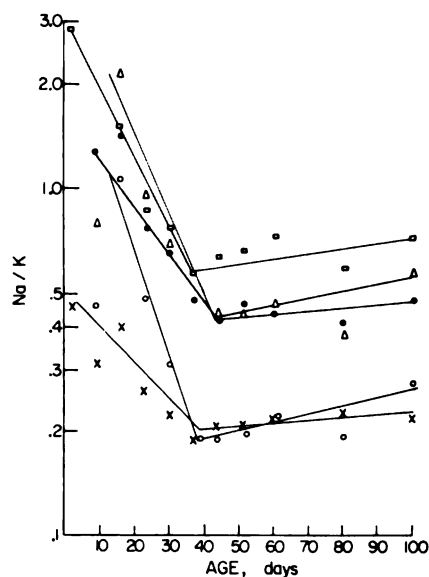
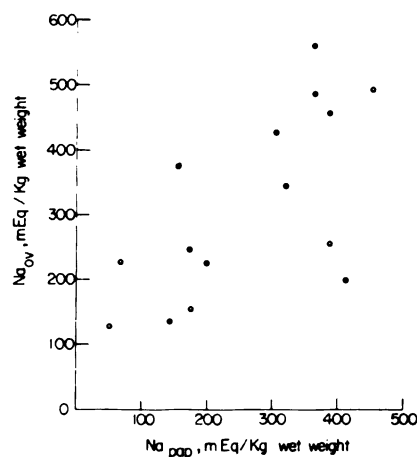


FIG. 2. Ratio of sodium to potassium tissue contents as a function of age. Points (●) represent heart; ○ = spleen; × = liver; △ = testis; and □ = kidney cortex. The same symbols are used in Fig. 4.

FIG. 3. Relationship between sodium content of ovary (Na_{OV}) and papilla (Na_{PAP}). Each point shows mean data from one animal.

that, since the development of renal regulation coincides with internal regulation, the cellular processes and renal homeostatic events are linked.

It is tempting to speculate that the linkage of regulatory development of electrolyte homeostasis is through maturation of transport processes. In support of this hypothesis, one can point to the increase in renal Na-K-ATPase which occurs during the prenatal

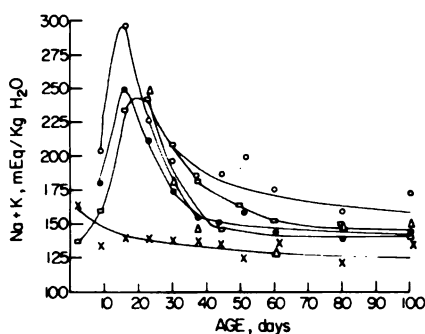


FIG. 4. Sum of sodium and potassium contents expressed as milliequivalents per kilogram of tissue water as a function of age. Symbols for tissues are the same as in Fig. 2.

period (11). The change in transport capacity, however, is dissociated from the developmental pattern of electrolyte balance and does not account for the fact that the sum of the cations decreases in the period examined (Fig. 4). In muscle and brain, such changes parallel an increase in tissue nitrogen (2, 3). At this point, one can only speculate as to what factors may be involved in reduction of the sum of Na and K. The increase in nitrogen (reflecting an increase in protein) may, however, cause changes in Donnan effects or may possibly reflect a change in the state of tissue electrolytes; i.e., different binding capacity (12).

The most surprising result obtained in these studies is the observation of high ovarian sodium which parallels papillary sodium (Fig. 3). The fact that papillary sodium is high is well known (6) and readily explained on the basis of the countercurrent system of the papilla. No comparable system is known for the ovary. It will be of interest to determine the mechanism which underlies high ovarian sodium and what its functional role may be.

Summary. Electrolyte and water contents of heart, liver, kidney, spleen, ovaries, and testes were analyzed in rats between 2 and 100 days of age. Tissues were dried to constant weight to measure water content. Electrolyte content was determined using emission spectrophotometry. At all times, the liver contained the least amount of water; the kidney, spleen, and heart had an intermediate water content which were approximately equal to each other; the testes and ovaries contained the highest content of

water. Water contents of the liver, heart, spleen, and kidney fall from 2 to 23 days of age and then remain constant at adult levels. The water contents of testes and ovaries remain relatively constant throughout the period studied. Tissue Na content of the heart, liver, kidney, spleen, and testes is high relative to K in the early postnatal period and decreases with age, reaching a minimum at 37–44 days of age. The most unexpected result of this study is the finding that the Na content of the ovary parallels that of the kidney papilla during all the periods examined. In both these tissues, Na content is low at 16 days of age and is higher thereafter. These results demonstrate a dissociation between maturation of water and electrolyte balance.

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Novel Chromogenic Substrate for Assaying Glucocerebrosidase Activity (39546)

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A recently synthesized phosphorylcholine derivative of 2-hexadecanoylamino-4-nitrophenol which structurally resembles naturally occurring glycolipid, sphingomyelin, was employed as a substrate for the lysosomal enzyme sphingomyelinase (sphingomyelin phosphodiesterase, EC 3.1.3.12). Displaying a sufficient specificity, this synthetic substrate analog was successfully used in the enzymatic diagnosis of Niemann-Pick disease, a familial metabolic disorder characterized by a deficiency of sphingomyelinase (3).

Based on this successful application, it was of interest to investigate the properties of α -glucopyranoside and β -D-galactopyranoside derivatives of 2-hexadecanoylamino-4-nitrophenol. These two glycosides are substrates of glucocerebrosidase and galactocerebrosidase. These glycolipids accumulate in lysosomal disorders, Gaucher's disease and Krabbe's disease, due to deficiencies in enzymes that normally degrade them, glucocerebrosidase- β -glucosidase (4) and galactocerebrosidase- β -galactosidase, respectively (5).

In the synthesis of 2-hexadecanoylamino-4-nitrophenyl- β -D-galactoside, the first synthetic substrate for galactocerebrosidase requiring specificity was developed. It was shown that this galactoside was readily hydrolyzed by tissue extracts such that its own release reflected the respective levels of galactocerebrosidase- β -galactosidase in the tissue of other β -galactosidases. Recently it was used in the clinical diagnosis and carrier detection of Krabbe's disease.

The present study describes the synthesis and enzymatic hydrolysis of 2-hexadecanoylamino-4-nitrophenyl- β -D-glucopyranoside and compares its properties to other substrates of glucocerebrosidase.

Materials and methods. 2,3,4,6-Tetra-O-acetyl- α -D-glucopyranosyl bromide was

purchased from Sigma Chemical Co. Cutscum was obtained from Fisher Chemical Co. Sodium taurocholate was purchased from Difco or Sigma Chemical Co. Silica gel G plates were supplied by Analtech, Inc. The compounds on the layer were visualized by charring with ammonium bisulfate according to Gal (7). Optical rotations were measured with a Perkin-Elmer 141 polarimeter at the 589-nm sodium line. Infrared spectra were obtained with a Perkin-Elmer 621 infrared grating spectrophotometer on KBr disks (1.0 mg of sample/300 mg of KBr). Visible absorption was measured on a Beckman ACTA MVI spectrophotometer. Melting points were taken on a Thomas-Hoover melting point apparatus and are corrected.

Enzymatic hydrolysis. Routine assays of β -glucosidase activity with 3 mM hexadecanoylamino-4-nitrophenyl-glucopyranoside were incubated in 50 mM citrate-phosphate buffer, pH 6.0, with 0.84% Cutscum and tissue extract in a final volume of 0.2 ml. Following incubation at 37° for 1 hr, the reaction was terminated by the addition of 0.1 ml of 1.0 M glycine-NaOH, pH 10.5. The suspension was clarified by the addition of 1.0 ml of ethanol followed by centrifugation at 2000g for 10 min. The clear supernatant was read at the absorption maximum of 415 nm. The extinction coefficient of the hydrolyzed product, the sodium salt of 2'-hydroxy-5'-nitrohexadecanilide, at this wavelength was determined to be 15,000. Glucocerebrosidase (8), 4-methylumbelliferyl- β -glucosidase (9) and *para*-nitrophenyl- β -glucosidase (10) were assayed as previously described.

Human spleen homogenates were prepared as 20% extracts (w/v) of previously frozen tissue in 20 mM citrate-phosphate buffer, pH 6.0, with 0.2% Cutscum and 1% sodium taurocholate. Following homogenization in a Waring Blendor, the suspension was centrifuged at 49,000g for 15 min and

the clear supernatant was used in the above assays.

The synthesis of 2-hexadecanoylamino-4-nitrophenyl- β -D-glucoside [3]. (Fig. 1).

2-Hexadecanoylamino-4-nitrophenyl-2,3,4,6-tetra-O-acetyl- β -D-glucopyranoside [2]. The commercially available 2,3,4,6-tetra-O-acetyl- α -D-glucopyranosyl bromide [1] was purified by washing an ethereal solution of this compound (15 \times , v/w) three times with 1 N sodium bicarbonate. After drying for 24 hr over anhydrous calcium chloride, the solvent was removed. The residue was recrystallized from (15 \times , v/w) of isopropyl ether; mp, 88–89°.

The bromide, [4] 8.22 g (20 mmoles), and the sodium salt of 2'-hydroxy-5'-nitrohexadecanilide (1, 2), 8.28 g (20 mmoles), were dissolved in 200 ml of acetone. After 6 hr at 25° the solution was refluxed for 24 hr and filtered from sodium bromide. The yellowish filtrate containing about 6% unreacted sodium salt was acidified with 0.2 ml of 6 N hydrochloric acid and evaporated. The residue was partially dissolved in 200 ml of ether and filtered from the starting material. The precipitates were washed with 20 ml of ether and the volume of the filtrate was reduced to about 50 ml and again filtered. The ether was evaporated and the residue was recrystallized from 500 ml of ethanol. Yield of [2], 9.4 g (65%); mp, 94–95°; $[\alpha]_D^{25} = -57.5^\circ$ (c 1.0 in acetone). On thin-layer chromatography in chloroform-methanol (99:1) it had $R_f = 0.49$ (acetobromoglucose had $R_f = 0.65$; compound [1] had $R_f = 0.22$).

Anal. Calcd for $C_{36}H_{54}N_2O_{13}$ (722.84): C, 59.82; H, 7.53; N, 3.87. Found: C, 59.51; H, 7.60; N, 3.94.

The above described reaction was repeated with 50% excess of 2,3,4,6-tetra-O-acetyl- α -D-glucopyranosyl bromide. The yield could not be improved.

2-Hexadecanoylamino-4-nitrophenyl- β -D-glucopyranoside [3]. To 2-hexadecanoylamino-4-nitrophenyl-2,3,4,6-tetra-O-acetyl- β -D-glucopyranoside [2], 7.22 g (10 mmoles), in 250 ml of distilled tetrahydrofuran was added 6 ml of a 0.5 N solution of sodium methylate in methanol (3 mmoles). The solution was kept for 1 day at room temperature. It was then stirred with 10 g of Dowex 50W-X8 (100–200 mesh) ion-exchange resin for 1 hr and filtered. The resin was washed with an additional 20 ml of tetrahydrofuran. The solvent was evaporated and the residue was recrystallized from 125 ml of methanol. Yield, 4.5 g (81%); mp, 148–149°. One gram of this product was recrystallized from 100 ml of methanol to which about 10 μ l of 1 N hydrochloric acid was added (for discoloration). The yield was 700 mg; mp, 152–153°; $[\alpha]_D^{25} = -59.3^\circ$ (c 1.0 in acetone). The infrared spectrum displayed bands at 3400 (O–H stretch), 2905, 2850 (alkane), 1670 (amide), 1520, 1335 (nitro), 1200 (phenol. weak), and 1070 cm^{-1} (primary alcohol). Thin-layer chromatography was carried out in chloroform-methanol-water (50:10:1); the compound had $R_f = 0.4$.

Anal. Calcd for $C_{28}H_{46}N_2O_9$ (554.69): C, 60.63; H, 8.36; N, 5.05. Found: C, 60.43; H, 8.35; N, 4.93.

A 2 mM solution of [3] in acetone buffer (1:1) solution showed less than 3% decomposition during 24 hr (25°) between pH 2 and 10. (potassium carbonate, borate, and hydroxide and potassium chloride-hydrochloric acid buffers, concentration: 5 mM). When [3] was heated with 2 N sodium hydroxide-acetone (1:1) for 2 hr at 100°, the sodium salt of 2'-hydroxy-5'-nitrohexadecanilide was formed displaying λ_{max} at 415 nm ($\epsilon = 15,000$) (2).

Results and discussion. The 2-hexadecanoylamino-4-nitrophenyl- β -D-glucoside was

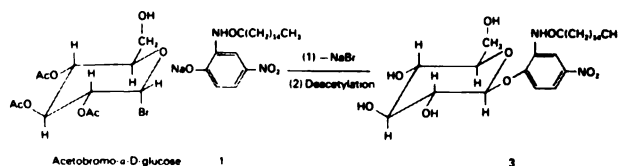


Fig. 1. Formulae and flow diagram for the synthesis of 2-hexadecanoylamino-4-nitrophenyl- β -D-glucopyranoside.

lyzed by crude human spleen extracts highly purified human placental glucosidase (8) at a pH optimum of 6.0. Kinetic characterization of this hydrolysis is summarized in Table I. This catalyzed hydrolysis is specific for human β -glucosidase since bitter almond β -glucosidase will not hydrolyze the glucoside (Table II). In contrast to two other synthetic glucosides, 4-*phumbelliferyl*- β -D-glucoside and *para*-nitrophenyl- β -D-glucoside which have been employed for the assay of glucocerebrosidase, this specificity since they are not hydrolyzed by the plant enzyme (Table II). With this newly synthesized β -glucoside we can detect and quantitate the deficiency of glucocerebrosidase in Gaucher's disease (Table III). Studies are underway to evaluate the action of leukocyte and cultured fibroblast extracts on this substrate in order to test its application further in the clinical diagnosis and carrier detection of Gaucher's disease. Hexadecanoylamino-4-nitrophenyl- β -glucoside represents a newly synthesized

TABLE I. KINETIC ANALYSIS OF 2-HEXADECANOYLAMINO-4-NITROPHENYL- β -D-GLUCOPYRANOSIDE AND GLUCOCEREBROSIDASE HYDROLYSIS BY PURIFIED HUMAN PLACENTAL GLUCOCEREBROSIDASE.

Parameter	Synthetic glucoside	Glucocerebrosidase
K_m	2.74	0.065
Turnover number	16	1000
nmol/hr/mg protein		
Optimum	6.0	6.4

TABLE II. HYDROLYSIS OF β -GLUCOSIDES BY HUMAN AND PLANT β -GLUCOSIDASE.

Substrate	Placental glucocerebrosidase (μ moles hydrolyzed/hr/mg protein)	Bitter almond β -glucosidase (μ moles hydrolyzed/hr/mg protein)
Hexadecanoylamino-4-nitrophenyl- β -D-glucoside	16	0
Glucosylceramide	400	0
4- <i>phumbelliferyl</i> - β -D-glucoside	20	15
4-Nitrophenyl- β -D-glucoside	40	25

TABLE III. HYDROLYSIS OF 2-HEXADECANOYLAMINO-4-NITROPHENYL- β -D-GLUCOSIDE (HNG) BY NORMAL AND GAUCHER'S SPLEEN EXTRACTS.

Spleen sample	Glucocerebrosidase ^a		HNGase ^b	
	(nmol/mg of protein/hr)	(% of control)	(nmol/mg of protein/hr)	(% of control)
Normal	43.0	—	0.72	—
Normal	41.0	—	0.75	—
Gaucher	3.8	9.0	0.12	16.3
Gaucher	3.8	9.0	0.05	6.8
Gaucher	4.0	13.5	0.11	14.9
Gaucher	3.9	9.2	0.07	9.5

^a Assays performed with 20 μ l (0.20 mg of protein) of spleen homogenate.

^b Assays performed with 100 μ l (1.0 mg of protein) of spleen homogenate.

glycoside which can be used in the assay of glucocerebrosidase. Although not as active as the natural substrate, it can be employed more readily because of the easily detectable chromogenic product formed as a result of its enzymatic breakdown. Its specificity over other synthetic β -glucosides favors its use in the diagnosis of Gaucher's disease.

Summary. 2-Hexadecanoylamino-4-nitrophenyl- β -D-glucopyranoside was synthesized. This compound structurally resembles glucocerebroside and is a specific substrate for glucocerebrosidase. This specificity offers advantages over other artificial substrates and the compound can readily be used in the detection of enzyme deficiency in extracts of spleens of patients with Gaucher's disease.

We wish to thank the Analytical Service Unit, National Institute of Arthritis, Metabolism and Digestive Diseases, NIH, for the elemental analyses.

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Somatostatin Inhibition of Glucagon-Stimulated Adenosine 3'-5'-Monophosphate Accumulation in Isolated Hepatocytes¹ (39547)

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Somatostatin, or somatotrapin release inhibitor factor is a small polypeptide found in the hypothalamus (1) and in the pancreatic islets of Langerhans (2). This small peptide has been shown to inhibit the secretions of growth hormone (1, 3, 4), thyroid stimulating hormone (5, 6) from the anterior pituitary gland and insulin from the endocrine pancreas. Studies with somatostatin *in vitro* produced similar results with inhibition of secretions of growth hormone, thyroid stimulating hormone (10), insulin, and glucagon (11-13). Somatostatin not only affects endocrine gland function but has also been shown to inhibit glucagon stimulated glycogenesis and glycogenolysis in isolated hepatocytes (11). The mechanism for these inhibitory effects is at present unknown. The nucleotide adenosine 3'-5'-cyclic monophosphate (cyclic AMP) has been implicated in the secretion of hormones as well as having a role in hepatic glycogenesis and gluconeogenesis. In the present study, we have investigated the effect of somatostatin on glucagon-stimulated cyclic AMP accumulation in isolated rat hepatocytes in an effort to elucidate the mechanism(s) by which somatostatin acts at the cellular level.

Materials and Methods. Male, well-fed Sprague-Dawley rats (150-200 g) were used throughout these studies. Hepatocytes were prepared by the collagenase perfusion technique (14). Approximately 50-70 mg of cells were incubated in 3.0 ml of Umbreit-25 mM bicarbonate buffer (15) aerated at 37° and 90 oscillations/min. Hepatocytes with or without somatostatin were incubated for 5 min before the addition of glucagon. After addition of glucagon incubation was continued for a fur-

ther 5 min, after which time 1 ml of 20% trichloroacetic acid was added, and the cells were homogenized for 15 sec using a Brinkman Polytron. The homogenate was centrifuged, and the cyclic AMP content of the supernatant was quantitated using the previously described method of Moxley and Allen (16).

Results. The effect of various concentrations of somatostatin on basal and glucagon-stimulated hepatocyte cyclic AMP accumulation are shown in Fig. 1. Somatostatin at concentrations of 0.50 to 8.0 µg/ml caused significant inhibition of cyclic AMP accumulation in hepatocytes stimulated with either 10^{-8} or 10^{-7} M glucagon. However, in the complete absence of glucagon, somatostatin at concentrations of 0.50 to 2.0 µg/ml caused a small but significant increase in cyclic AMP accumulation. A similar finding was also observed for hepatocytes exposed to a low concentration of glucagon, 10^{-12} M, and a somatostatin concentration of 2.0 µg/ml.

Discussion. These studies indicate that somatostatin can inhibit glucagon-stimulated cyclic AMP accumulation in hepatocytes. The rather paradoxical finding of somatostatin increasing cyclic AMP levels in hepatocytes not exposed to glucagon or as in one case when glucagon concentration was low, 10^{-12} M, are difficult to interpret. However, such findings would suggest that somatostatin is behaving as a partial agonist. Evidence in support of this concept is based on a structural relationship between somatostatin and glucagon, both of which have the same four amino acids, Thr-Phe-Thr-Ser occurring in the same sequence.

Studies by other groups (10, 17) have shown that somatostatin inhibits basal and prostaglandin E_1 or thyrotropin releasing hormone-stimulated cyclic AMP accumulation in rat pituitary glands. In isolated rat islets of Langerhans, Efendic *et al.* (18) has

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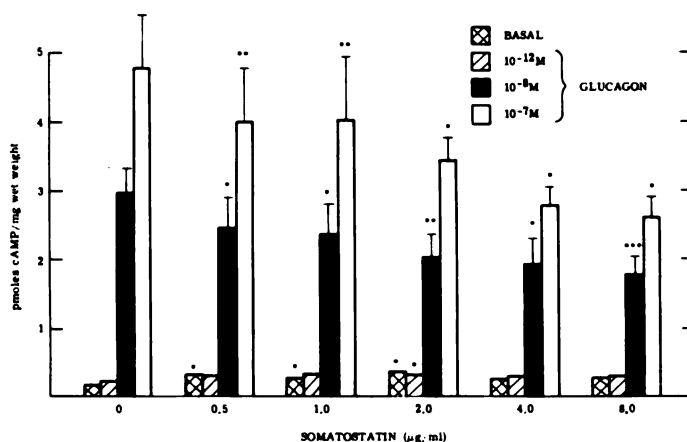


FIG. 1. The effect of increasing concentrations of somatostatin on basal or glucagon-stimulated cyclic AMP accumulation in isolated rat hepatocytes. The bars represent the mean \pm SEM for six to eight observations from four rats. Statistical analysis was done by Student's *t* test using paired differences. * Represents $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

shown that somatostatin inhibits glucose-stimulated accumulation of cyclic AMP. In each of these studies a decrease in cyclic AMP levels was concomitant with a decrease in secretion of hormone from the endocrine gland. In earlier studies from this laboratory (11) glucagon-stimulated glycogenolysis and gluconeogenesis could be inhibited by concentrations of somatostatin (0.2 to 2.0 $\mu\text{g/ml}$) similar to those used in the present study. Although cyclic AMP has been implicated in glycogenolysis and gluconeogenesis (19, 20), the exact interrelationship between cyclic AMP and these two processes is still unknown. The work of Pilkis *et al.* (21) has shown that the addition of glucagon or of cyclic AMP to isolated rat hepatocytes increased lactate incorporation into glucose. Both the cyclic AMP and glucagon effects on gluconeogenesis were dependent on the presence of extracellular calcium ion; when there was a deficiency of calcium, gluconeogenesis was inhibited. This same group of workers also showed that glucagon-stimulated accumulation of cyclic AMP was independent of extracellular calcium. These findings would perhaps suggest that calcium plays a more important role in gluconeogenesis than does cyclic AMP. Investigators using isolated rat islets of Langerhans (12) and perfused pancreas (22) have shown that the inhibitory effects of somatostatin on glucose-stimulated insulin secretion could be overcome by increas-

ing the extracellular calcium ion concentration.

Data presented in these present studies support our earlier findings (11) that somatostatin is capable of direct action on hepatocytes and strengthen the concept that somatostatin is capable of direct action on other tissues resulting in alterations of normal cellular functions. However, whether somatostatin affects cellular function by inhibiting cyclic AMP accumulation or by altering translocation of calcium within cells still remains to be elucidated.

Summary. The cyclic form of somatotropin release inhibitor factor over a concentration range from 0.50 to 8.0 $\mu\text{g/ml}$ was found to inhibit glucagon-stimulated cyclic AMP accumulation in isolated rat hepatocytes.

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Effect of Microsomal Enzyme Inducers on the Biliary Excretion of an Exogenous Load of Bilirubin in Newborn Rats¹ (39548)

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Neonatal physiological jaundice is a condition seen in some newborn children which is characterized by high plasma levels of unconjugated bilirubin. If unconjugated bilirubin reaches high plasma levels or if the bilirubin is displaced from the plasma proteins to which it is bound, bilirubin will enter the central nervous system and produce a type of brain damage termed kernicterus. It has been demonstrated that when phenobarbital is given to pregnant women before delivery, there is a reduction in the plasma bilirubin levels of the infants (1-3). However, if the neonates were not exposed to phenobarbital until after birth, the treatment was less effective (3-5) and the best treatment appeared to be when the neonate was exposed to the drug both *in utero* and during the first few days of life.

While most laboratory animals do not develop neonatal physiological jaundice, many have been shown to have an impaired ability to excrete an exogenous load of bilirubin in comparison to the adult (6, 7). This effect appears to be due both to an impaired conjugation and excretion.

We have demonstrated that the biliary excretion of a number of drugs is impaired in newborn rats (8-10). The biliary excretion of one of these drugs, ouabain, can be stimulated to develop at an earlier age by the administration of microsomal enzyme inducers (11). Spironolactone and pregnenolone-16 α -carbonitrile were more effective in this regard than phenobarbital. Therefore, it was of interest to determine (i) if the biliary excretion of an exogenous load of bilirubin is impaired in the newborn rat as has been shown in other species and (ii) if

there is a difference in the ability of various microsomal enzyme inducers to enhance the development of the processes responsible for its excretion.

Materials and methods. Rats of various ages were used throughout the study. The rats were born in our laboratory and were produced by mating untreated Simonsen Sprague-Dawley rats. The mother and offspring were kept in "shoebox cages" for 1 month before separation. The rats had free access to food and water at all times.

Rats of 1, 6, 11, 16, 21, or 26 days of age were pretreated with the various microsomal enzyme inducers for 4 days. Phenobarbital sodium (PB; Merck and Company, Rahway, N. J.) was administered ip (50 mg/kg) in propylene glycol (5 ml/kg) to rats over 15 days of age. Rats less than 15 days of age were given 33 mg/kg of phenobarbital (3.3 ml/kg), because the higher dose decreased growth rate. Rats were also pretreated with spironolactone (S, 75 mg/kg; G.D. Searle and Company, San Juan, P. R.), pregnenolone-16 α -carbonitrile (PCN, 75 mg/kg; The Upjohn Company, Kalamazoo, Mich.) or 3-methylcholanthrene (3-MC, 20 mg/kg; Eastman Kodak Company, Rochester, N. Y. in propylene glycol (5 ml/kg). Control rats were given propylene glycol (5 ml/kg). On the fifth day, [³H]bilirubin was administered (30 mg/kg) into the distal portion of the femoral vein.

[³H]bilirubin (unconjugated) was prepared by a modification of the method of Ostrow *et al.* (12). δ -Aminolevulinic acid hydrochloride (3,5-³H (N)) (New England Nuclear, Boston, Mass.) was administered to anesthetized rats (urethane, 800 mg/kg ip) and bile was collected overnight in a test tube covered with aluminum foil and immersed in an ice bath. The [³H]bilirubin was isolated from the bile as previously described (12). To determine if the ³H excreted into bile after iv administration of

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bilirubin is representative of the amount of bilirubin excreted into bile, bilirubin was administered to adult, anesthetized, bile-duct-cannulated rats. Bile collected at 10, 20, 30, and 45 min after administration to control rats and rats that had been pretreated with microsomal enzyme inducers. The amount of bilirubin excreted into the bile quantitated by radiochemical techniques was found to be $99.8 \pm 1.9\%$ of that quantitated by the diazo colorimetric method (13).

Twenty-four hours after the last pretreatment with a microsomal enzyme inducer, bilirubin was mixed with nonlabeled bilirubin (30 mg/kg) (K & K Laboratories, New York, N. Y.) and was prepared in an isotonic solution containing 0.5 g of Na_2CO_3 , 0.52 g of NaCl /100 ml of distilled water (1/kg) and injected into the distal portion of the femoral vein. Twenty minutes after the rats were sacrificed and the small intestine was removed. As a measure of the biliary excretion of bilirubin, the small intestine was homogenized with 3 ml of saline in a Kaman Polytron homogenizer (Luzern, New York). This method was used to estimate biliary excretion because it was not feasible to cannulate the bile duct of small rats and measure biliary excretion directly. This technique for estimating biliary excretion was verified by comparing the amount of bilirubin in the small intestine of bile-duct-ligated and control rats 20 min after iv administration of [^3H]bilirubin; the concentration in bile-duct-ligated rats was 9.3% of that in controls.

In this study, the amount of [^3H]bilirubin excreted into the small intestine was quantitated not only at 20 min after administration but also at 5, 10, and 30 min. In this study a blood sample was also taken by cardiac puncture under light ether anesthesia. Heparin was used as the anticoagulant. The rats were sacrificed and both the liver and small intestine were removed.

After centrifugation, aliquots of plasma (50 μl), intestinal homogenate (500 μl) and liver (approx 500 mg) were oxidized with a Packard Model 306 Tri-Carb oxidizer, and radioactivity was estimated with a Packard Model 3330 Tri-Carb scintillation spectrometer (Packard

Industries, Downers Grove, Ill.). Quenching was determined by using automatic external standardization. All values are expressed as milligram equivalents of bilirubin.

The data were compared by an analysis of variance. When the analysis indicated that a significant difference existed, the means of the treated groups were compared to the control mean by Student's *t* test (14).

Results and discussion. Figure 1 demonstrates the amount of [^3H]bilirubin excreted into the bile within 20 min after administration of [^3H]bilirubin to control and phenobarbital-treated rats. Rats 15 days of age and younger were less efficient in excreting the bilirubin into the bile than were the older rats. Thus, the hepatic processes responsible for the biliary excretion of bilirubin are not mature in the newborn rat, similar to what has previously been shown for the guinea pig (6) and rabbit (7).

Pretreatment with phenobarbital tended to increase the biliary excretion of bilirubin (Fig. 1) but this was not statistically significant at any age. The ineffectiveness of phenobarbital in increasing the biliary excretion of bilirubin may be due to the relatively low dose administered due to its pharmacological and toxicological effects; rats less than 15 days of age were given 33 mg/kg and from 15 to 30 days of age, 50 mg/kg. In studies with adult rats, 75 mg/kg of phenobarbital is usually given.

Figure 2 depicts the effect of spironolactone and the hepatic disposition of bilirubin in rats of various ages. Pretreatment with spironolactone increased the biliary excretion of bilirubin in rats of all ages examined except the 30-day-old rats.

The effect of pregnenolone-16 α -carbonitrile (PCN) on the development of hepatic excretory process is shown in Fig. 3. Rats at all ages pretreated with PCN excreted significantly more bilirubin into the bile than controls. Pretreatment of rats with 3-methylcholanthrene had no effect on the biliary excretion of bilirubin (Fig. 4).

While the first four figures demonstrate the effect of the four microsomal enzyme inducers on the amount of bilirubin excreted into the bile within 20 min of its administration, Fig. 5 depicts the plasma concentra-

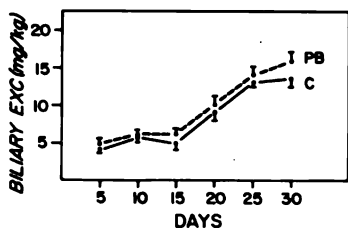


FIG. 1. The effect of pretreatment with phenobarbital (33 or 50 mg/kg) once a day for 4 days on the amount of bilirubin excreted into the intestine 20 min after administration (30 mg/kg iv) in rats of various ages. Each point represents the mean \pm SE of three to eight rats.

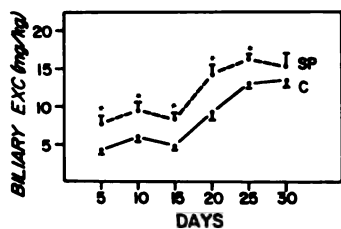


FIG. 2. The effect of pretreatment with spironolactone (SP, 75 mg/kg) once a day for 4 days on the amount of bilirubin excreted into the intestine 20 min after administration (30 mg/kg iv) in rats of various ages. Each point represents the mean \pm SE of three to eight rats. Asterisks indicate the values that are significantly different from controls (C).

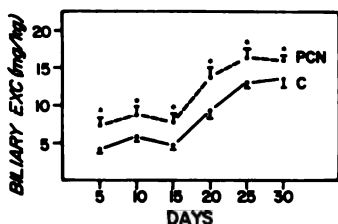


FIG. 3. The effect of pretreatment with pregnenolone-16 α -carbonitrile (PCN, 75 mg/kg) once a day for 4 days on the amount of bilirubin excreted into the intestine 20 min after administration (30 mg/kg iv) in rats of various ages. Each point represents the mean \pm SE of three to eight rats. Asterisks indicate the values that are significantly different from controls (C).

tion, the amount in the liver, and the amount excreted into the bile at various times after administration of the [3 H]bilirubin in 15-day-old control and PCN-pretreated rats. PCN increased the disappearance of bilirubin from the plasma. It also increased the amount in the liver at 5 and 10 min after administration. At the lat-

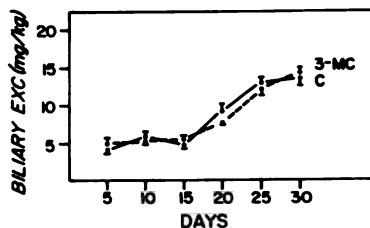


FIG. 4. The effect of pretreatment with 3-methylcholanthrene (3-MC, 20 mg/kg) once a day for 4 days on the amount of bilirubin excreted into the intestine 20 min after administration (30 mg/kg iv) in rats of various ages. Each point represents the mean \pm SE of three to eight rats. C, control.

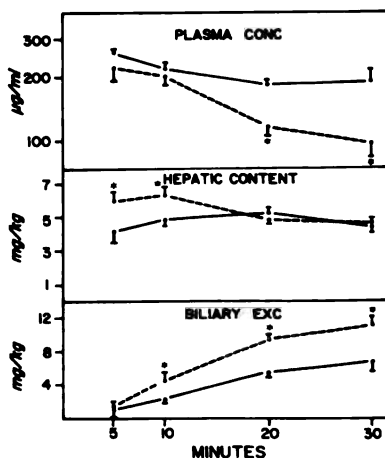


FIG. 5. Effect of PCN pretreatment (once a day for 4 days) on the hepatic disposition of bilirubin in 20-day-old rats. [3 H]bilirubin was administered iv (30 mg/kg). Each value represents the mean \pm SE of six to eight rats. Solid lines represent the controls and dashed lines represent the PCN-pretreated rats. Asterisks indicate the values that are significantly different from controls.

ter time intervals no difference was observed, probably due to the larger amount excreted into the bile of the PCN-treated rats. No difference in the biliary excretion of bilirubin was observed at 5 min, but at the 10-, 20-, and 30-min time intervals the PCN-treated rats excreted more bilirubin into the bile than controls.

A definite difference in the ability of various microsomal enzyme inducers to enhance the plasma clearance and biliary excretion of bilirubin has been demonstrated. Why this difference exists and why the newborn is less efficient in clearing bilirubin is

not clear. The low amount of ligandin in the newborn (15) has been suggested as an explanation. However, phenobarbital is more effective in increasing the amount of ligandin in the liver than is spironolactone and PCN (16) but it is less efficient in enhancing bilirubin clearance in the newborn. 3-MC has previously been shown to be a better inducer of hepatic bilirubin glucuronyltransferase activity than other microsomal enzyme inducers (17) yet it had no effect on the clearance of bilirubin in the newborn rat. However, spironolactone and PCN stimulate the excretion of exogenous chemicals to a greater extent than does phenobarbital, and 3-MC is without effect (18-20), which parallels the effect seen in newborn rats to bilirubin. Thus, indirectly, it would appear that the increased clearance of bilirubin after spironolactone and PCN in the newborn rat is probably more dependent on the increased excretion of bilirubin than it is on the amount of ligandin or glucuronyltransferase. Possibly the rate-limiting step in the clearance of bilirubin in the newborn rat is the transfer from liver to bile, as has been reported for the adult rat (21, 22).

Summary. The biliary excretion of administered unconjugated bilirubin in the newborn rat was demonstrated to be immature. Newborn rats were treated with microsomal enzyme inducers to determine whether they would enhance the overall hepatic excretory system responsible for the biliary excretion of bilirubin. 1-, 6-, 11-, 16-, 21-, or 26-day-old rats were pretreated for 4 days with phenobarbital, pregnenolone-16 α -carbonitrile, spironolactone, or 3-methylcholanthrene. On the fifth day, [^3H]bilirubin was administered (30 mg/kg). Twenty minutes after the amount of bilirubin excreted into the intestine was determined. Spironolactone and pregnenolone-16 α -carbonitrile enhanced the biliary excretion of bilirubin but phenobarbital and 3-methylcholanthrene were without effect.

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Structural Changes in Glyceraldehyde-3-Phosphate Dehydrogenase Isolated from Temperature-Acclimated Rainbow Trout (*Salmo gairdneri*)¹ (39549)

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It is well documented that the activity and kinetics of various enzymes are changed as a result of temperature acclimation (1). There is evidence that the differences reported may be due to the functional significance of isoenzymes in thermal acclimation (2, 3). We have previously reported kinetic and thermodynamic differences in glyceraldehyde-3-phosphate dehydrogenase (G3PDH) isolated from warm, 15° (G3PDH(W)), and cold, 5° (G3PDH(C)), acclimated rainbow trout, *Salmo gairdneri* (4).

There are also data which show that, although temperature-dependent interconversions may take place which result in altered kinetics, the components may not be separable by electrophoretic or electrofocusing analyses (5). It is assumed that the observed kinetic changes in our case are due to a temperature-dependent conformational change which is stable through enzyme isolation procedures.

In order to establish that stable conformational changes did occur as a result of temperature acclimation, we have measured several physical parameters of purified G3PDH. Our coenzyme binding studies establish a difference in the enzyme as a result of temperature acclimation. The spectroscopy data indicate a conformational difference.

Materials and methods. Rainbow trout (*Salmo gairdneri*) were obtained from the Federal Fish Hatchery at Leetown, W. Va. They were maintained in aquaria at either 15° (warm) or 5° (cold). They were fed every other day with food supplied by the hatchery.

After 3 weeks, fish were sacrificed and

G3PDH was purified from the lateral muscle following the method of Cori *et al.* (6). All reagents contained 10⁻³ M dithiothreitol. The resulting product moved as a single band in polyacrylamide-gel electrophoresis using amido black as a stain.

The enzymes were assayed in a Beckman DB spectrophotometer at 340 nm with a recorder adjusted to 6 × 10⁻⁶ M NADH full scale. The reaction mixture contained 40 mM triethanolamine at pH 8.5, 5 mM Na₂HPO₄, 1.58 mM NAD, and glyceraldehyde phosphate (GAP) at concentrations ranging from 18.3 to 300 μM. The concentration of the D-isomer of GAP was determined by the reaction with triosephosphate isomerase and glycerolphosphate dehydrogenase which was measured spectrophotometrically. NAD was purchased from Pabst Laboratories, GAP from Sigma Chemical Company.

Electrophoresis was carried out in a Canco Model 1200 in a 4° refrigerator. The procedure followed that of Davis (7), except that gel polymerization was catalyzed by ammonium persulfate, and phenol red was used as a tracking dye. Gels of 6 and 8.5% cross-linkage were used. The cathode buffer was at pH 8.4 and the anode buffer at pH 7.5. Samples were electrophoresed until the tracking dye was within 0.5 cm of the bottom of the gel tube. Gels were removed from the tubes and stained with amido black (0.5 mg/ml in 15% acetic acid) for 2 hr. The gels were destained in 15% acetic acid.

Measurements of coenzyme binding were carried out on a Perkin-Elmer MPF-2 spectrofluorimeter. The excitation wavelength was 280 nm; the emission was measured at 350 nm (8). Fluorescence was measured with successive 10-μl additions of a 1 mM solution of NAD to 2.00 ml of a 2.4 μM solution of G3PDH in 0.01 M Tris-acetate buffer at pH 8.5. The procedure of Stockell (9) was used to analyze the NAD concentra-

¹ Supported in part by Research Grant 463, North Atlantic Treaty Organization.

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and the fraction bound to give the number of binding sites and the intrinsic dissociation constant.

Ultraviolet spectra of three warm and two cold preparations were recorded on a Cary spectrophotometer at room temperature with distilled water. Values of optical density were read from the spectra every 5 nm between 200 and 350 nm. The extinctions were calculated at each of these wavelengths using a molecular weight of 100,000. The extinctions at each wavelength were averaged for the three warm and two cold preparations. The resulting values were plotted on a Calcomp X-Y plotter. A difference spectrum was obtained by subtracting the average cold from the average warm extinction.

Two average spectra were each fitted with a model which assumed that the absorption at each wavelength was a linear combination of the absorption of tyrosine, phenylalanine, and tryptophan.

The matrix was set up using the following equation:

$$\text{Gyr}_\lambda (x_1) + \text{Phe}_\lambda (x_2) + \text{Trp}_\lambda (x_3) + e_\lambda,$$

at wavelength λ : E_λ = total extinction; Gyr_λ = extinction of tyrosine; Phe_λ = extinction of phenylalanine; Trp_λ = extinction of tryptophan; x_1 , x_2 , and x_3 = molar fractions of Tyr, Phe, and Trp, respectively, in G3PDH; and e_λ = error of estimate. The equation was solved by least squares, so as to obtain the best average solution of x_1 , x_2 , and x_3 from the spectrum and to minimize e_λ . The difference between G3PDH(W) and G3PDH(C) was obtained by subtracting the value of the number of moles of each amino acid in the cold enzyme from those in the warm enzyme. The value obtained corresponds to the difference in number of amino acids in a position to absorb radiation.

Circular dichroism (CD) spectra for each enzyme preparation were measured on a Cary Model 60 spectropolarimeter fitted with a Model 6001 CD attachment. The thermal acclimation of the enzymes was determined by assuming that the mean residue ellipticity at a given wavelength was due to a combination of the mean residue ellipticities of the mole fractions of α -helix, β -

sheet, and random coil forms (10). A matrix was set up with the equation:

$$\theta = x_1 A_\lambda + x_2 B_\lambda + x_3 R_\lambda + e_\lambda,$$

where at wavelength λ : θ = mean residue ellipticity in degrees square centimeters per decimole; A_λ , B_λ and R_λ = mean residue ellipticities of 100% α -helix, β -sheet, and random coil forms, respectively; e_λ = error in estimates. The best values for x_1 , x_2 , and x_3 were obtained by minimizing the error over a range of discrete wavelengths (10) which best indicate the α -helix, β -sheet, or random coil structures.

Protein concentration was determined by the method of Lowry *et al.* (11).

Results and discussion. Cold acclimation has no effect on the electrophoretic properties of G3PDH. Coelectrophoresis of a sample of G3PDH(W) and G3PDH(C) shows that the enzymes move as a single band in various gel cross-linkages. The single band indicates that both species have approximately the same size and molecular weight per charge ratio. G3PDH is an oligomeric enzyme (12) and from several sources has been found to contain four identical subunits in the active form (13), and there is no evidence showing that G3PDH contains any number other than four identical subunits. From electrophoresis data, it follows that temperature acclimation does not change the number of subunits.

The results of the NAD-binding experiments are shown in Fig. 1. Both the 15°C enzyme and the 5°C enzyme have three binding sites. This corresponds to the number of binding sites found in the rabbit muscle enzyme (8). The intrinsic association constants for NAD are $6.68 \times 10^4 \text{ M}^{-1}$ for G3PDH(W) and $12.3 \times 10^4 \text{ M}^{-1}$ for G3PDH(C). These values are averages of those obtained by duplicate measurements on each of two G3PDH preparations. It can be concluded that temperature acclimation affects NAD binding.

The interaction of G3PDH with NAD appears to be affected by the thermal history of the animal from which the enzyme was obtained. Greene and Feeney (14) have reported that the K_m values of NAD are affected by "cold-adaptation." They found the K_m to be $1.80 \times 10^{-5} \text{ M}$ for the rabbit

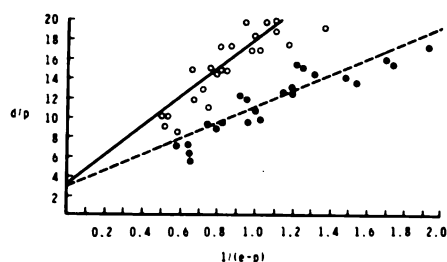


FIG. 1. NAD binding: d = concentration of NAD, e = enzyme concentration, p = fraction of sites bound times enzyme concentration. There are three NAD-binding sites on each enzyme. For the 15° enzyme $K_{\text{NAD}}^0 = 6.68 \times 10^4 M^{-1} \pm 13\%$; for the 5° enzyme $K_{\text{NAD}}^0 = 12.3 \times 10^4 M^{-1} \pm 10\%$.

muscle G3PDH and $7.62 \times 10^{-5} M$ for the "cold-adapted" fish muscle enzyme (*Disostichus mawsoni*).

The difference in the uv spectra of cold and warm G3PDH measured between 350 and 200 nm is shown in Fig. 2. This plot represents the difference between the average cold and warm spectra. Protein absorption in this portion of the spectrum is due to absorption of aromatic amino acids (15). Differences in this region can be correlated with differences in the positioning of tryptophan, phenylalanine, and tyrosine. Based on the difference spectrum, it can be seen that the cold enzyme has a greater absorption than the warm. This is probably due to the aromatic amino acids in the cold enzyme being in a better position to absorb radiation. The results of the calculated difference, shown in Table I, show the change in the number of each of the residues in a position to absorb radiation for each of the studied enzymes.

These results are compatible with published amino acid analyses of various species of G3PDH. Greene and Feeney (14) reported that for both the rabbit and the halibut G3PDH there are 40 tyrosines, 60 phenylalanines, and 16 tryptophans/mole. The spectral analysis in the present study shows that about one-half of the tyrosines and phenylalanines and one-fourth of the tryptophans are affected by temperature acclimation. These data, then, are consistent with the idea that a stable conformational change has occurred *in vivo* in the G3PDH as a result of temperature acclimation.

The CD spectra are shown in Fig. 3. The

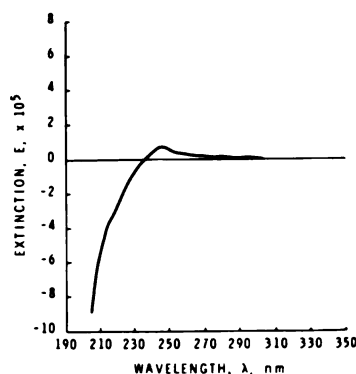


FIG. 2. G3PDH difference spectrum: Curve illustrates the difference when the extinction coefficient cold enzyme is subtracted from the extinction coefficient of warm enzyme.

TABLE I. DIFFERENCE IN UV-ABSORBING RESIDUES FOLLOWING THERMAL ACCLIMATION.

	(Moles of warm) (Moles of cold)
Tyrosine	19
Phenylalanine	26
Tryptophan	4

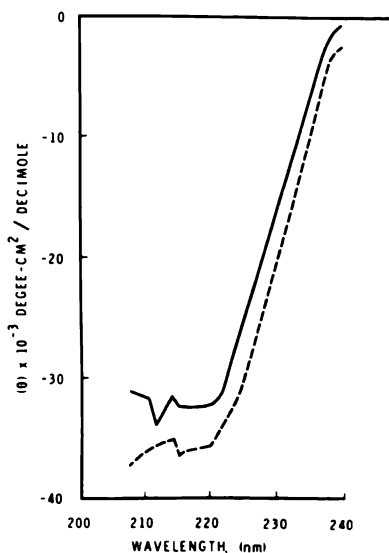


FIG. 3. CD spectra. (---), Average of 15° zymes; (—), average of 5° enzymes.

TABLE II. SECONDARY STRUCTURE OF G3PDH FOLLOWING THERMAL ACCLIMATION.

	α (%)	β (%)	Random coil (%)
15° enzyme	9.1	14.0	76.9
5° enzyme	11.1	13.0	75.9

results of the analysis are shown in Table II. The spectra obtained closely resemble those of carboxypeptidase A, chymotrypsin, and chymotrypsinogen (10). The 15 and 5° enzymes have approximately the same distribution of α helix, β sheet, and random coil. The similarity of the CD spectra of the 15 and 5° enzymes shows that there is probably not a large difference in the secondary structure of the two enzymes. The change is more likely a difference in the higher-order structure of the holoenzyme rather than a transition of helix to β structure or random coil.

The activity of G3PDH changes with temperature acclimation. We are concerned in this study with the mechanism by which this activity is affected. Changes in subunit structure are ruled out by electrophoretic studies. The similarity of the CD spectra, as well as the relative invariance in amino acid composition of the enzyme isolated from various species, further weakens the hypothesis of synthesis, *de novo*, of subunits with significantly different secondary structure.

It may be concluded, then, that temperature acclimation results in a change in the higher-order structure of the G3PDH which is stable through isolation procedures. This is supported by a difference in uv-absorption characteristics and NAD-binding properties between the warm and cold enzyme. In addition, immunological data from our laboratories (16) to be published elsewhere also support the idea of altered tertiary or quaternary structure.

Summary. Glyceraldehyde-3-phosphate dehydrogenase has been purified from rainbow trout acclimated to 15° (warm) and 5° (cold) and compared with respect to their electrophoretic properties, NAD-binding

behavior, and ultraviolet absorption. Both the warm and cold species of G3PDH show the same electrophoretic properties. They differ, however, in NAD-binding behavior and in ultraviolet-absorption properties. It was concluded that temperature acclimation brings about a conformational change in the higher-order structure of G3PDH which is stable through purification procedures.

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calorie	cal	millimeter	mm
centimeter	cm	milliosmole	mOsm
counts per minute	cpm	minute	min
cubic centimeter	cm ³	molal (concentration)	<i>m</i>
Curie	Ci	molar (concentration)	<i>M</i>
degree Celsius (Centigrade)	-°	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	dia	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	<i>N</i>
inch	in.	osmole	Osm
inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
intravenous	iv	percent	%
kilocalorie	kcal	picogram	pg
kilogram	kg	revolutions per minute	rpm
liter	spell out	second	sec
meter	m	specific activity	sp act
microliter	μl	square centimeter	cm ²
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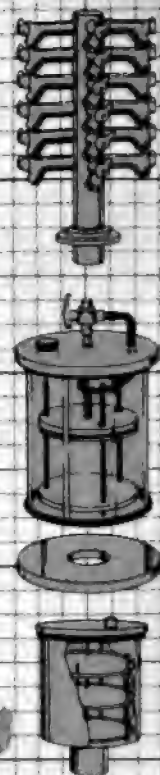
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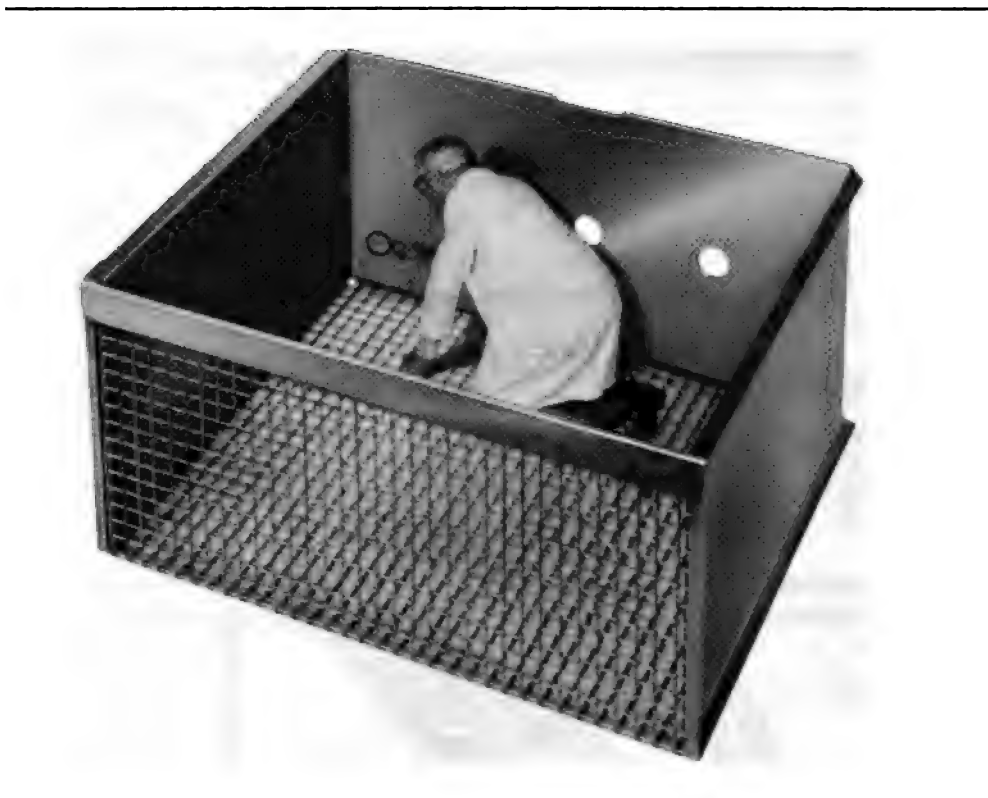
PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE

Volume 153, Number 3, December 1976

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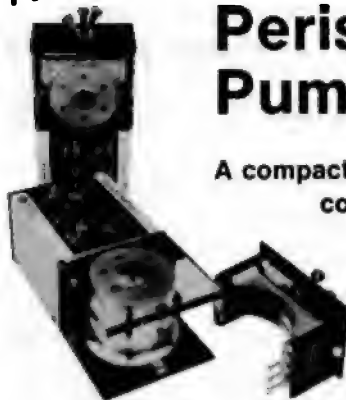
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Effect of Truncal Vagotomy on Lower Esophageal Sphincter Pressure and Response to Cholinergic Stimulation¹ (39550)

RICHARD H. HIGGS AND DONALD O. CASTELL

ology Division, Internal Medicine Service, and Clinical Investigation Service, Naval Regional Medical Center, Philadelphia, Pennsylvania 19145

Truncal vagotomy is commonly performed for treatment of peptic ulcer disease. Previous observations suggest that vagotomy does not alter resting lower esophageal sphincter (LES) pressure (1-3). The effect of truncal vagotomy upon the cholinergic response of the LES has not been determined. Cholinergic stimulation with bethanechol has been shown to increase sphincter pressure both in control and reflux patients (4). Further, cholinergic stimulation with bethanechol has been shown to have a therapeutic benefit in the treatment of esophageal reflux (5). Thus, any effect of truncal vagotomy on the LES response to cholinergic stimulation with bethanechol would have both physiologic and therapeutic implications.

In the present studies were performed to determine the effect of vagotomy on the cholinergic response of the LES in man and in a rat model.

Human studies. Patients studied in the present groups included 7 male patients (mean age 44 years) having had prior truncal vagotomy with pyloroplasty (V&P), 10 patients (mean age 41 years) having had truncal vagotomy and antrectomy (V&A), and 4 male patients (mean age 46 years) having had prior antrectomy (A). All patients had Billroth II procedure. The three groups consisted of patients for whom surgery had been required for complicated peptic ulcer disease. Ten healthy volunteers (mean age 34 years) with no history of surgery formed the control group. All procedures were intra-abdominal in approach, with sectioning of the vagal trunks as far distal as possible through the hiatus. The

presence or absence of a complete vagotomy in V&P and V&A patients was confirmed by the appropriate acid response to insulin-induced (0.2 unit/kg iv) hypoglycemia, as previously described (6).

Intraluminal pressure measurement. Three water-filled polyvinyl tubes, 1.4-mm i.d., transmitted intraluminal pressure to external transducers (Statham Series p 23), and pressure was recorded on a multichannel direct-writing recorder (Hewlett-Packard 7700 Series). Intraluminal pressures were recorded through three lateral openings 1.3 mm in diameter placed 5 cm apart. The tube was passed into the stomach and slowly withdrawn until it was positioned with the middle aperture recording maximal LES pressure just below the respiratory inversion point. This position was verified at least once every 10 min throughout the entire study period. The system was infused with distilled water at a constant rate of 0.42 ml/min.

Sphincter pressure was recorded in millimeters of mercury with mean resting intragastric pressure used as zero reference. Average pressure was determined from the recording for each minute of the test. The basal pressure represented the mean value for 15 such determinations. Sphincter response was determined from the mean pressure level of the five highest consecutive 1-min values after stimulation. Student's *t* test was utilized for evaluating statistical significance between mean pressure levels.

Bethanechol stimulation. The effect of graded doses of bethanechol (0.01, 0.02, 0.04, and 0.08 mg/kg sc) was studied in all subjects. Significant side effects prohibited use of larger doses. On separate days after a 15-min basal period, a single dose was given randomly in a blinded fashion as to dose, and LES pressure was monitored for an additional 45 min.

Supported by the Bureau of Medicine and Surgery, Clinical Investigation Program No. 5-05-530R.

Reprint requests and correspondence to Dr. Donald O. Castell, National Naval Medical Center, Bethesda, Maryland 20814.

Cat studies. Because significant side effects prevented us from using larger doses of bethanechol in humans, we also used an experimental animal to obtain a complete dose-response curve. Five adult male cats, averaging 4 kg in body weight, received the following doses of bethanechol iv before and 8 weeks after bilateral transabdominal truncal vagotomy: 3.0, 6.0, 12.0, 25.0, and 50.0 $\mu\text{g}/\text{kg}$. Thirty minutes after being anesthetized with ketamine hydrochloride (20 mg/kg im), LES pressure was measured by a series of slow "pull-throughs" of the recording orifice from stomach to esophagus at 1-min intervals. Basal pressure represented the mean of the highest two pressures. After iv administration of bethanechol, "pull-throughs" were done every minute for 20 min and the peak response represented the two highest consecutive values. All manometric tracings were coded and interpreted blindly following completion of all studies. Student's *t* test was again utilized for evaluating statistical significance between mean pressure levels.

Results. Human studies. Basal LES pressure. Basal LES pressure for controls and V&P patients is shown in Fig. 1A. Mean basal pressure for controls (13.0 ± 1.5 mm Hg, \pm SE) was not significantly different from that of V&P patients (11.6 ± 1.6 mm Hg). In addition, no significant difference was noted between mean basal pressure of V&A patients (12.6 ± 1.6 mm Hg) and A patients (12.5 ± 2.3 mm Hg), as shown in Fig. 1B.

Bethanechol. LES pressure changes fol-

lowing sc bethanechol in both controls and V&P patients are shown in Fig. 2A. The mean increase in pressure for V&P patients was significantly greater than that of the controls at the 0.02, 0.04, and 0.08-mg/kg dose ($P < 0.01$, 0.05, and 0.01, respectively). The maximal mean LES pressure change was 42.0 ± 2.2 mm Hg for V&P patients and 27.3 ± 2.3 mm Hg for controls. Figure 2B illustrates the pressure changes in V&A and A patients following sc bethanechol. At all four doses the response of the V&A group was significantly ($P < 0.02$) greater than that of A patients. The maximal mean LES pressure change was 34.7 ± 2.3 mm Hg for V&A patients and 12.0 ± 2.0 mm Hg for A patients.

Cat studies. Basal pressure. As shown in Fig. 3, the mean LES basal pressure in the cats before vagotomy (32.5 ± 4.7 mm Hg) was not significantly different from that after vagotomy (37.2 ± 3.6 mm Hg).

Bethanechol. The LES response to graded iv doses of bethanechol for the five cats before and after vagotomy is shown in Fig. 4. The mean postvagotomy responses to 3.0 (13.4 ± 1.5 mm Hg) and 6.0 $\mu\text{g}/\text{kg}$ (34.0 ± 5.0 mm Hg) were significantly greater ($P < 0.05$) than those found prior to vagotomy (7.5 ± 3.5 and 18.3 ± 3.8 mm Hg). However, the maximal mean LES responses before and after vagotomy were not statistically different, although occurring at a different dose. Prior to vagotomy, the mean maximal response (30.2 ± 2.9 mm Hg) occurred with the 25- $\mu\text{g}/\text{kg}$ dose. After vagotomy, the mean maximal response of $34.0 \pm$

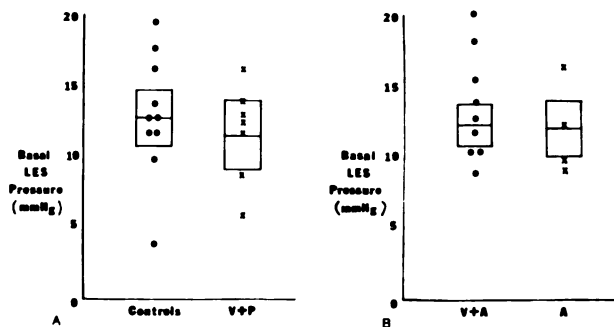


FIG. 1: Basal lower esophageal sphincter (LES) pressure for controls and patients having vagotomy and pyloroplasty (V&P) on the left (A) and for patients with vagotomy and antrectomy (V&A) and patients with antrectomy (A) on the right (B). Each point represents the mean of at least nine values for each individual. Vertical boxes are \pm SE.

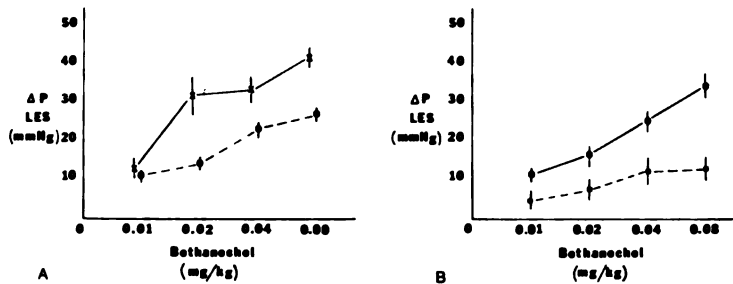


Fig. 2. Dose-response curves for change in lower esophageal sphincter (LES) pressure (ΔP) against log dose of bethanechol for controls (closed circles) and patients having vagotomy and pyloroplasty (x's) on the left (A) or patients with vagotomy and antrectomy (open circles) and patients with antrectomy (squares) on the right. Points indicate mean values and vertical lines \pm SE.

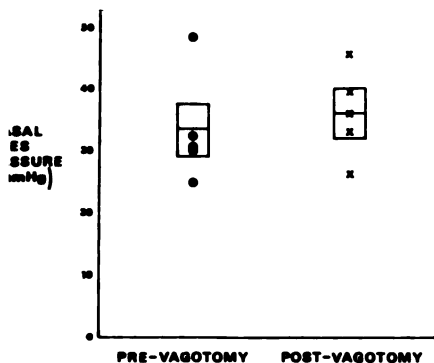


Fig. 3. Basal lower esophageal sphincter (LES) pressure for cats prevagotomy and postvagotomy. Each box represents the mean of at least five values for at least five cats before and after vagotomy. Vertical boxes are

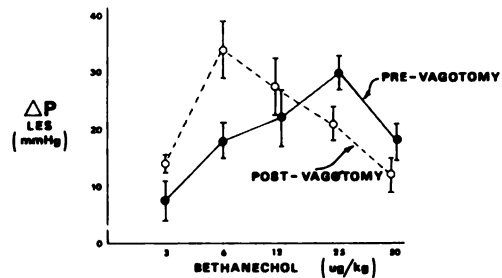


Fig. 4. Dose-response curves for change in lower esophageal sphincter (LES) pressure (ΔP) against log dose of iv bethanechol for cats prevagotomy and postvagotomy. Points indicate mean value and vertical lines \pm SE.

mm Hg occurred with the 6.0- μ g/kg

Discussion. Our studies show that the pressure response to bethanechol in cats with truncal vagotomy (V&P and A) is significantly increased when compared to the response of those without vagotomy (controls and A). These results indicate that vagotomy produces an increased sensitivity of the LES to cholinergic stimulation. This increased sensitivity to cholinergic stimulation following truncal vagotomy was observed in the cat.

Although it is of interest to speculate concerning the cause of the increased sensitivity of the sphincter to cholinergic stimulation, it is not readily apparent. Classically sympathetic fibers were thought to be innervated in the vagus (7). Thus, vagotomy might produce denervation of cholinergic

pathways resulting in the increased sensitivity to cholinergic stimulation, as has been postulated to occur in achalasia (8-10). Studies with cervical vagotomy in the opossum, however, have failed to demonstrate that any stimulatory efferent cholinergic pathways were carried in the vagal nerves to the sphincter (11). Thus, interruption of parasympathetic pathways per se in the vagus is not a likely cause of this enhanced responsiveness of the LES to bethanechol after vagotomy.

Certain types of denervation supersensitivity have been postulated; however, to be nonspecific, that is, when innervation of an end organ is interrupted, the end organ may demonstrate increased responsiveness to any agent which ordinarily effects a response (12). Therefore, the increased response to bethanechol after vagotomy does not necessarily indicate that a cholinergic pathway per se has been interrupted. Thus, one might speculate that truncal vagotomy may interrupt noncholinergic neural path-

ways to the sphincter which have been shown to be carried in the vagus (13, 14), an interruption of which, because of nonspecificity of denervation supersensitivity, might produce the increased response to bethanechol.

The absence of a significant difference in basal LES pressure between patients with and without vagotomy supports previous studies in man showing that truncal vagotomy does not alter resting sphincter tone (1-3), as well as studies with cervical vagotomy in the opossum (15). This observation is of interest in light of the simultaneous supersensitive response of the LES to cholinergic stimulation. If cholinergic mechanisms or agents which act via cholinergic mechanisms do affect basal LES pressure such as has been postulated in achalasia, it seems reasonable to expect vagotomy to have increased resting sphincter tone. Therefore, on the basis of our studies in man and cats, it seems unlikely that cholinergic mechanisms have any major effect in maintaining basal LES pressure.

Summary. The effect of truncal vagotomy on the lower esophageal sphincter pressure and the response to cholinergic stimulation have been studied in humans as well as an animal model, the cat. Vagotomy was found to result in enhanced responsiveness to cholinergic stimulation. However, basal sphincter pressure was unchanged. These observa-

tions suggest that cholinergic mechanisms do not have a major effect in maintaining basal LES tone.

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Calcitonin, Serotonin, and Parafollicular Cell Granules during the Hibernation Activity Cycle in the Bat (39551)

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small, dense, smooth, membrane-en-
granules of mammalian thyroid para-
lar cells have been recognized as stor-
anules containing calcitonin (1, 2).
eptide hormone inhibits bone resorp-
nd thus lowers plasma calcium (3). A
ial variation in the number and mor-
y of these granules in bats (4-8) and
hibernating mammals (9) suggests
ecretory activity slows prior to hiber-
, ceases during hibernation, and re-
during arousal.

afollicular cells of bats also store the
amine serotonin (2, 10). Tritiated ser-
, synthesized from its tritiated precur-
-hydroxytryptophan ([5-³H]HTP), is
ed in the small granules of the cell
The serotonin content of the bat thy-
aries in accordance with the morpho-
ly defined seasonal secretory cycle of
arafollicular cell. It rises when the
es accumulate in early hibernation
radually falls as granules are resorbed
; mid and late hibernation.

se observations have led to the sug-
n that the two hormones, calcitonin
rotonin, are stored in the same sub-
storage granules. If this hypothesis
ect, seasonal covariation of the thyro-
content of the two hormones is to be
ed.

is report correlates thyroidal calci-
plasma calcium, thyroidal serotonin,
arafollicular cell morphology through
tivity-hibernation cycle in the bat.

Materials and methods. Animals. Fully
male and female bats of the species
; *lucifugus* were used. All animals
aptured in their natural habitat. Ac-
its were captured in May, September,
October. Hibernating bats were col-
in November, December, January,

and February. The latter were placed
gently, while in the cave where they were
hibernating, into containers with ice, trans-
ported to the laboratory, and kept at 4° for
at least 24 h before use. During this period,
the animals were watched for signs of physi-
cal activity. Those exhibiting such activity
were not used.

Sample preparation. At each seasonal
time point, some animals were anesthetized
by ether inhalation and blood was drawn by
cardiac puncture. Blood from four to six
bats was pooled for determination of plasma
calcium. Other animals were decapitated
and their thyroids were quickly excised and
trimmed. Some glands were fixed in
buffered 6.25% glutaraldehyde (pH 7.3)
for 4 hr, washed, and processed for electron
microscopy as described elsewhere (7). The
remaining glands (between 40 and 60) were
weighed and pooled into two separate sam-
ples (10 for serotonin analysis; the remain-
der for calcitonin). The pooled thyroids
were quickly frozen by immersion in liquid
nitrogen and were kept at -20° until pro-
cessed.

Measurement of serotonin. The spectro-
fluorometric method of Snyder *et al.* (12)
was used. Serotonin, extracted from an acid
homogenate of the thyroids, was reacted in
aqueous solution at neutral pH with ninhy-
drin. The excitation and emission spectra of
the intensely fluorescent product were ob-
tained at 380 and 490 nm, respectively, with
an Aminco Bowman spectrofluorometer
and were compared with those of serotonin-
creatinine sulfate standards. Results were
expressed as micrograms of serotonin per
gram of thyroid wet weight.

Measurement of calcitonin. Pooled sam-
ples of 30 to 50 thyroids were processed for
measurement of calcitonin at each seasonal
time point by a rat bioassay method used
previously in this laboratory (13). The
glands were homogenized at 4° in 0.1 N

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HCl. The homogenate was centrifuged in the cold at 10,000 rpm for 20 min. The supernatant was collected, pH was adjusted to 4.0, and the protein content was estimated (14). Holzman albino male rats, averaging 100 g in weight, were fasted overnight and used for bioassay of hypocalcemic activity in the supernatant against the Medical Research Council (MRC) calcitonin standard B. The bioassay was designed as a two-dose factorial assay (15) with high and low points obtained for each homogenate. Blood was drawn by cardiac puncture 1 hr after injection of the supernatant and plasma calcium was determined by flame emission spectrophotometry (13). At least five rats were used for each point. Statistical evaluation was performed as described earlier (13, 15). Potency estimates were expressed as mean MRC milliunits per milligram of protein (MRC mU/mg) of the respective supernatant.

Bat plasma calcium was measured in pooled bat blood as described above by flame emission spectrophotometry and expressed in milliequivalents per liter (mequiv/liter). Significance of differences was tested by the Student's *t* distribution.

Results. Calcitonin levels. A seasonal profile of bioassayable calcitonin in bat thyroid

is shown in Fig. 1. The corresponding calcitonin levels are listed in Table I. Calcitonin levels in thyroid glands obtained late in hibernation (February) were similar to those observed in thyroids of active bats (May and September). The mean level of thyroidal calcitonin in active bats ranged between 30 and 43 MRC mU/mg. A marked increase in levels was observed in glands collected in early hibernation (November and December). The mean level observed during this period was two- to threefold that of active animals and ranged between 60 and 123 MRC mU/mg. In spite of a relative increase in the standard error estimate, indicating greater heterogeneity among pooled preparations, the difference between these values and those observed in late hibernation and during the active phase of the annual life cycle is clearly significant.

Plasma calcium levels. Pooled plasma calcium levels during the hibernation period revealed that relative hypocalcemia occurs in midhibernation (November; Fig. 1) with plasma calcium levels of 4.2 ± 0.2 mequiv/liter compared to 4.8 ± 0.2 mequiv/liter ($P < 0.01$) observed during the active phase of the annual life cycle. Hypocalcemia during hibernation in bats has also been observed by Riedesel (16).

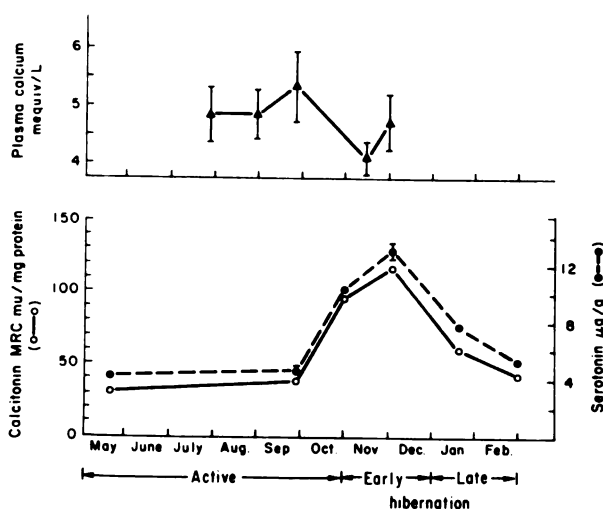


FIG. 1. A seasonal profile of bat thyroidal calcitonin, serotonin, and plasma calcium. (Upper panel): Plasma calcium in mequiv/liter (mean \pm SE). (Lower panel): Calcitonin potency estimates in bat thyroid extracts, expressed in mean MRC milliunits per milligram of protein. Open circles, solid line. Serotonin content expressed in micrograms per gram of thyroid wet weight. Filled circles, broken line. The bars at the September- and December-time points indicate the standard error.

TABLE I. CALCITONIN AND SEROTONIN CONTENT OF BAT THYROID.

h	Calcitonin (MRC mU/mg) ^a Mean \pm SE	Serotonin (μ g/g) ^b Mean \pm SE ^c
er	30 \pm 4.50	4.05
er	42 \pm 5.04	4.15 \pm 0.1
er	93 \pm 11.60	10.20
er	123 \pm 24.6	12.68 \pm 0.28
y	60 \pm 8.40	4.67
y	40 \pm 5.50	5.20

^a Hypocalcemic activity expressed in MRC millir milligram of protein of gland extract.
^b Serotonin in micrograms per gram of gland wet

Multiple (three) determinations were performed at seasonal time points: September and December. Mean and SE shown are for these time points.

serotonin levels. The seasonal changes in total serotonin content in the bat are shown in Fig. 1 and the corresponding values are listed in Table I. Thyroidal serotonin levels in bats ranged between 4 and 4.25 μ g/g in early hibernation, a threefold increase is observed with serotonin levels at 13 μ g/g. This increase subsided by the time when serotonin content is back at hibernation levels.

Morphology of parafollicular cell secretory granules. The morphological appearance of parafollicular cell secretory granules varied in the active phase, pre- or early hibernation phase, hibernation, and arousal are depicted in Fig. 2a-d. During the pre- or early hibernation phase of the annual cycle (May to early September), only small granules with a diameter between 0.1 and 0.2 μ m were observed (Fig. 2a). They are usually spherical, contain a solid dense core, and are bounded by a smooth membrane.

In the pre- or early hibernation (September through late October), large dense intracellular granules are found in addition to all dense secretory granules. These intracellular granules are bounded by a membrane dotted with ribosomes and reach diameters up to 5 μ m (Fig. 2b). Large intracellular granules are absent later in hibernation.

At this time, however, the small, secretory granules lose their solid core and often contain membranes or ring-like figures (Fig. 2c). The granules in this stage do not exhibit uptake of tritium-labeled hydroxytryptophan (11). At arousal,

small, solid, dense granules are again found in parafollicular cells (Fig. 2d).

Discussion. A parallel seasonal variation in thyroidal calcitonin content and thyroidal serotonin content is reported in the present study. The thyroidal content of both hormones increases at the start of hibernation and decreases as hibernation proceeds. A rise in thyroid calcitonin during hibernation has also been found in the ground squirrel (17). The changes in hormonal content described above are closely related to the morphological changes in parafollicular cell secretory granules. Thus, in early hibernation these granules accumulate and large dense granules appear in the cisternae of the rough endoplasmic reticulum. These suggest a slowing of intracellular transport of secretory material from the rough endoplasmic reticulum to the Golgi apparatus. During midhibernation, autophagy of preformed granules is commonly observed. The number of granules decreases and the matrix of residual granules becomes less electron dense (4, 6, 8). By prearousal, new granules appear (6) and arousal is accompanied by signs of exocytosis of granular contents (6).

It seems likely that the increase in calcitonin content of the thyroid in early hibernation is due to slowed or inhibited release of the hormone from the parafollicular cells. Relative hypocalcemia, actually observed during hibernation (this report; 16) is undoubtedly one factor responsible for and contributing to this. It is reasonable to assume that the absence of a dietary source of calcium during hibernation could explain a relative hypocalcemia. In fact, evidence for increased parathyroid activity (18) and increased skeletal resorption to the point of skeletal demineralization in hibernating bats (19) indicates the extent of corrective mechanisms induced by the hypocalcemia. The inhibition of intracellular transport and release of calcitonin from parafollicular cells during hibernation, clearly facilitates the effect of these corrective mechanisms toward homeostasis of calcium during hibernation. Thus, we would postulate that parafollicular and parathyroid cells have reciprocal roles and act synergistically in the regulation of calcium metabolism during the seasonal life cycle of mammalian hibernators.

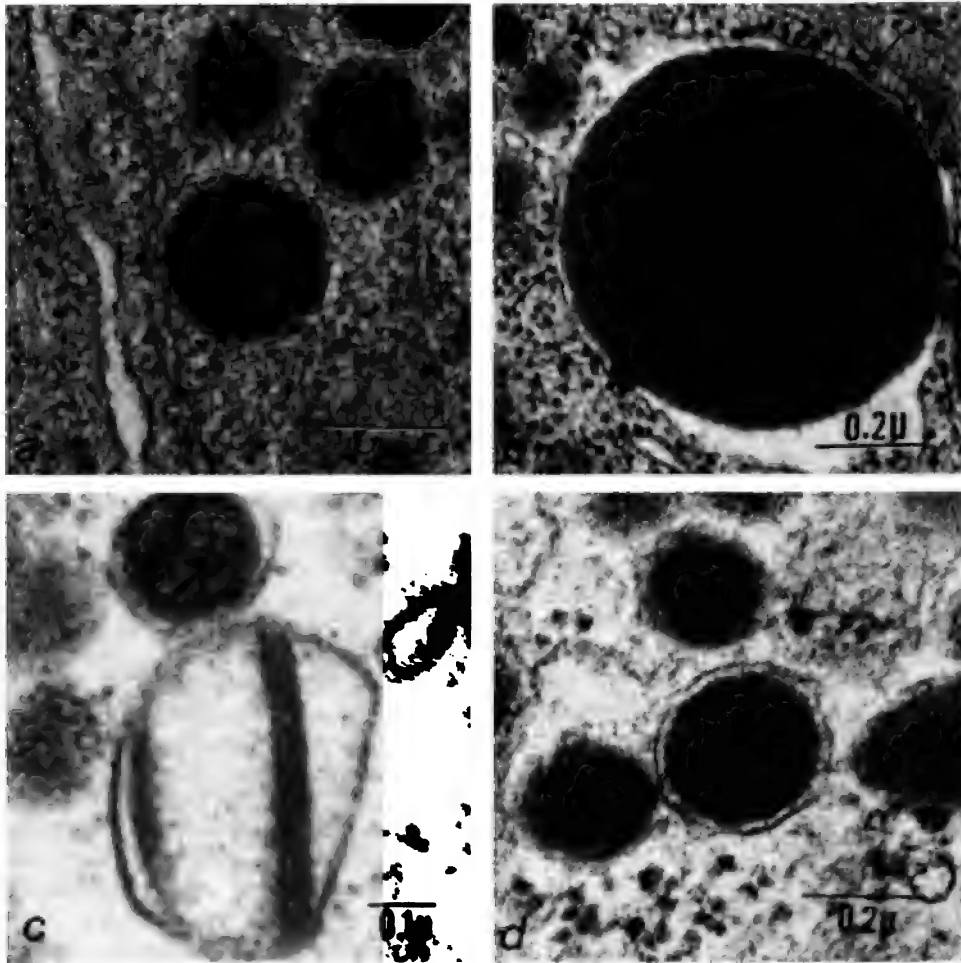


FIG. 2. A profile of seasonal ultrastructural changes in bat thyroid parafollicular cell granules. (a) Secretory granules during the active phase of the life cycle. Electron-dense core bounded by a smooth membrane. Magnification, $\times 80,000$. (b) Large intracisternal granules seen during prehibernation and early hibernation. Arrows indicate the ribosome-studded limiting membrane. Magnification, $\times 76,000$. (c) Resorbing secretory granules seen during mid- to late hibernation. Note gradual loss of electron-dense core. Magnification, $\times 90,000$. (d) Secretory granules as seen at the arousal phase of the annual life cycle. Magnification $\times 80,000$.

The present data, taken together with earlier observations (2, 10), support the hypothesis that serotonin and calcitonin are stored in the same parafollicular cell granules. The coincidence of storage would seem to ensure that both hormones would be released together by exocytosis. Serotonin release from parafollicular cells by calcium, the natural stimulus for calcitonin release, has recently been demonstrated (20).

Since serotonin is rapidly cleared from the circulation through uptake and inactivation by platelets, liver, and pulmonary endothe-

lial cells (21), its role, once released from thyroid parafollicular cells, is likely to be a local one. Biogenic amines have been suggested as endogenous stimulators of thyroid follicular cells (22-24). Serotonin, specifically, has been implicated in the action of thyrotropin on follicular cells (25). Similarly, in bat thyroid, serotonin has been proposed to act as a local activator "messenger" between the parafollicular "storage" cells and the follicular cells (26). The latter are capable of serotonin uptake and inactivation (26). Thus, changes such as hypo-

hypercalcemia might indirectly affect thyroid hormone secretion as well as calcitonin secretion.

Brain serotonin and the serotonergic neurons of the nuclei of the median raphe have been implicated in the control of hibernation (27). The presence of serotonin in the bat thyroid and the seasonal variation in its content suggest that thyroidal serotonin may play an important role in the hibernation process. The definition of such a role remains to be determined.

Summary. A seasonal covariation in calcitonin and serotonin content of bat thyroid has been found. Whereas the mean level of thyroidal calcitonin in active bats ranged between 30 and 48 MRC mU/mg of protein, a two- to threefold increase in this level was observed prior to and during early hibernation. Associated with this increase, a relative hypocalcemia was observed. In late hibernation, thyroidal calcitonin level returned to the range observed in active bats. A parallel seasonal profile was found for bat thyroid serotonin content, which varied from a mean of 4.1 $\mu\text{g/g}$ during the active phase of the annual life cycle to a peak close to 13 $\mu\text{g/g}$ observed in early hibernation. The seasonal covariation of calcitonin and serotonin reflects ultrastructural changes observed in the secretory granules of thyroid parafollicular cells during the annual life cycle of the bat.

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Intrarectal Infection of Guinea Pigs with the Agent of Guinea Pig Inclusion Conjunctivitis¹ (39552)

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Chlamydial infection of the human genital tract is a topic of current concern (1). A well-known venereal disease caused by a chlamydial agent is lymphogranuloma venereum (LGV). The association of chlamydiae with human urethritis (2) and cervicitis (3) is a subject of great interest. Chlamydiae have been recovered from the rectum as well as from the genital tract (4). Organisms causing human genital infection as well as ocular disease are classified in Group A (*Chlamydia trachomatis*). In general, chlamydial infections occurring in other animals are caused by organisms in Group B (*Chlamydia psittaci*).

In our laboratory, we have developed the guinea pig as an animal model system for the investigation of chlamydial genital infections using the agent of guinea pig inclusion conjunctivitis (Gp-ic) discovered by Murray in 1964 (5). In the natural host, this organism causes a mild conjunctivitis. It has been shown that experimentally infected female guinea pigs develop vaginitis and cervicitis and are capable of transmitting ocular infection to newborns (6). The male guinea pig can be infected experimentally by the intraurethral route, and subsequent transmission of genital infection to females has been demonstrated (7).

In our former studies, leg lesions were observed in male guinea pigs inoculated intraurethrally (8). These lesions may now be accounted for by the use of Innovar-vet as an analgesic tranquilizer since this drug has been shown to cause self-mutilation in guinea pigs (9).

In view of the possible significance of rectal infection in man caused by chlamydiae, we have investigated experimental infection of guinea pigs by the intrarectal route with the agent of Gp-ic. The results are presented in this report.

Materials and methods. Methods for preparation of inoculum from Gp-ic-infected yolk sac suspensions, collection of vaginal and conjunctival scrapings for smears stained with Giemsa, detection of antibodies by indirect immunofluorescence have been described previously (6, 7).

Guinea pigs. Mature Hartley strain guinea pigs were obtained from Simonsen Laboratories, Inc., Gilroy, Calif. All animals were pretested in our laboratory for antibodies to Gp-ic. Conjunctival and vaginal smears were also examined for Gp-ic inclusions, and no evidence was obtained for previous or current infection. Animals were caged individually for these experiments.

Intrarectal inoculation. All animals were fasted overnight before inoculation. A sterile vinyl tubing attached to a 23-gauge needle on a 1.0-ml syringe was inserted about 1.5 in. (3.8 cm) into the rectum. Each animal received 0.25 ml of inoculum containing approximately 2×10^6 ELD₅₀. Leakage from the rectum occurred in most instances so that exact volume retained was not determined.

Rectal swabs and smears. A dental spatula was inserted into the rectum and scraped over the intestinal wall. Sterile cotton swabs were inserted into the rectum and gently rotated. The swabs were placed in 1.0 ml of SPG (sucrose-potassium-glutamate) containing streptomycin (2.5 mg/ml) and vancomycin (0.5 mg/ml). Samples were frozen at -70°C until used for inoculation of chick embryos.

Isolation of Gp-ic from intestinal seg-

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ents. Animals were anaesthetized with ether and exsanguinated by cardiac puncture. A rectal swab was obtained and the peritoneum was opened using sterile technique. The intestine was severed at the most distal point within the pelvis and dissected on a sheet of sterile aluminum foil. Segments were removed at intervals of approximately 3 in. (7.6 cm) and the contents were pressed onto the foil. One part of each segment was placed in SPG and frozen at -70°C for homogenization and inoculation to chick embryos. An adjacent segment was collected for histopathology.

Results. The pattern of recovery of Gp-ic after inoculating male and female guinea pigs intrarectally is shown in Fig. 1. Gp-ic is recovered from all animals on Day 7 postinoculation and from 10 of 14 on Day 14, indicating that all animals were successfully infected. By Day 14, six of eight males were still shedding the agent whereas only one female of seven was positive. After this period, Gp-ic was recovered from only a few animals. One male (no. 317) was still positive on Day 35, but a specimen collected on

Day 42 yielded negative results. In several animals, a sporadic pattern of isolation was observed and it is not known if this type of response was due to inadequate collection of specimens or sporadic shedding of the agent.

Attempts were made to detect Gp-ic inclusions in rectal smears stained with Giemsa. Most of the specimens were inadequate for evaluation but in two animals (male no. 319, female no. 347) sufficient cellular material was obtained and Gp-ic inclusions were observed in cells. The guinea pigs were also examined for overt clinical response with negative results. Rectal temperatures were followed for 7 days postinoculation with no elevations observed.

As shown in Table I, antibody responses were detected following intrarectal inoculation with Gp-ic. All animals had been pretested prior to inoculation and all sera were negative at a dilution of 1:10. Antibodies were detected in all animals except male no. 319 which remained negative throughout the course of the experiment. Titers were generally in the range of 10–20, and by Day 42, 5 of the 15 animals yielded negative

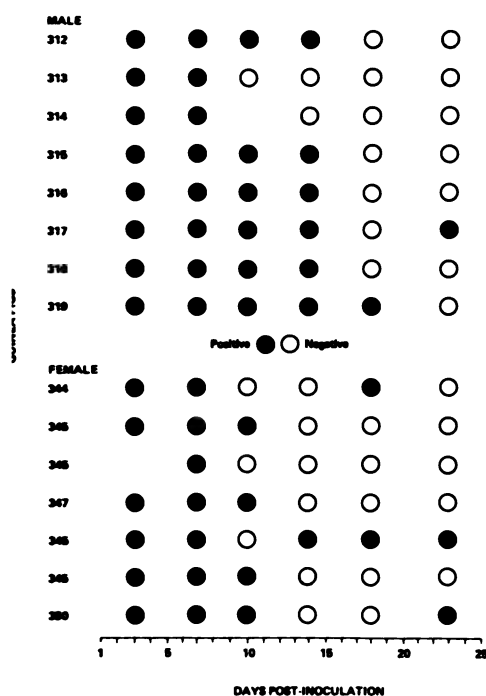


FIG. 1. Recovery of Gp-ic agent from guinea pigs inoculated intrarectally. Rectal swabs were collected and inoculated into 7-day-old chick embryos.

TABLE I. ANTIBODY RESPONSE IN GUINEA PIGS INOCULATED INTRARECTALLY WITH Gp-ic.

Guinea pigs	Day postinoculation		
	21	42	77
Male			
312 ^a	20 ^b	40	10
313	20	10	<10
314 ^a	10	<10	<10
315 ^a	10	10	<10
316	10	<10	<10
317	10	<10	<10
318 ^a	20	10	<10
319	<10	<10	<10
Female			
344	20	ND ^c	<10
345 ^a	20	20	<10
346	10	<10	<10
347 ^d	<10	10	<10
348	40	80	10
349	20	40	<10
350	20	40	10

^a Conjunctival smear positive for chlamydial inclusions.

^b Antibody titer determined by indirect immunofluorescence; see Materials and Methods.

^c Not done.

^d Vaginal smear positive for chlamydial inclusions.

results. Only 3 of the 15 animals had detectable antibodies by Day 77.

In order to learn the extent of spread of Gp-ic in the intestinal tract following intrarectal inoculation, groups of male and female guinea pigs were inoculated and sacrificed on selected days and the intestinal tract was dissected (see Materials and Methods). Rectal swabs from 16 of 20 animals tested on the day of sacrifice were positive for Gp-ic (Table II). Homogenates of rectal tissue yielded Gp-ic from 12 of the 21 animals. In considering the possible migration of the infection in the intestine, combination of results obtained from section nos. 4, 5, and 6 (Table II) revealed that Gp-ic was not isolated from these sites on either Day 3 or Day 7. However, from Days 10 to 21, the agent was recovered from these sites. It was not apparent that Gp-ic was uniformly distributed throughout the intestinal tract. Failure to recover the agent from adjacent sections may have been due to technical error or may indicate a patchy localization of the agent. None of the serum specimens

collected on date of sacrifice in this experiment yielded positive results for Gp-ic antibodies. Histologic sections of the intestinal segments did not reveal any obvious pathologic process and Gp-ic inclusions were not observed in superficial epithelium.

It was also of interest to examine the incidence of ocular and genital infections following intrarectal inoculation. In this study, a total of 8 animals of 35 tested yielded ocular smears that were positive for Gp-ic inclusions. Inclusions were also detected in vaginal smears of 2 of 16 females.

Discussion. Male to female transmission of Gp-ic by sexual contact has been documented under experimental conditions (7), but data have yet to be obtained for natural genital infection of either males or females. The demonstration of experimental intrarectal infection of guinea pigs with Gp-ic broadens the potential use of this *Chlamydia*-host system for the study of genital infections caused by these agents. No obvious clinical condition or histopathologic response following rectal infection was ob-

TABLE II. RECOVERY OF Gp-ic FROM INTESTINAL TRACT OF GUINEA PIGS INOCULATED INTRARECTALLY.

Guinea pig	Intestinal segments							
	Rectal ^a swab	Rectum	1 ^b	2	3	4	5	6
Day 3 M-352	+ ^c	+	-	-	C ^d	-	-	-
M-353	+	+	+	+	-	-	-	-
F-364	-	+	+	-	-	-	-	-
F-366	-	-	-	-	-	-	-	-
Day 7 M-354	+	+	-	-	-	-	-	-
M-355	+	-	-	-	-	-	-	-
F-367 ^e	+	+	C	+	-	-	-	-
F-368	+	+	+	-	-	-	-	-
Day M-356	+	-	-	-	-	-	-	-
10 M-357	-	+	-	-	-	-	+	-
F-369	+	-	-	-	-	-	-	-
F-379	+	+	-	-	-	-	-	+
Day M-359 ^f	+	-	-	+	-	-	+	-
14 M-360	+	+	-	+	-	-	-	-
F-371	C	+	+	-	+	+	+	-
F-372 ^g	+	-	-	-	-	-	-	-
Day M-358	+	+	-	-	-	-	-	-
17 M-361 ^h	+	-	-	-	+	-	-	+
M-362	+	-	-	-	-	-	-	-
F-374	+	+	-	-	-	-	ND ⁱ	-

^a Collected on day of sacrifice.

^b Most distal segment of colon from abdomen.

^c Specimens were inoculated into chick embryos via yolk sac route.

^d Contaminated.

^e Vaginal smear positive for chlamydial inclusions.

^f Conjunctival smear positive for chlamydial inclusions.

^g Not done.

. Our experiments showed that shed-
f Gp-ic could be demonstrated in
and females for at least 10 days fol-
intrarectal inoculation. After this
he incidence was apparently lower in
s than in males. Recovery of Gp-ic
pper segments of the intestinal tract
es the potential for continued infec-
n this regard, it is also possible that
ination of food and cages with fecal
al led to infection by fecal-oral route.
ita are available on this potential

une response was documented in ani-
nocolated intrarectally. Antibody ti-
ere somewhat lower than those ob-
in females inoculated intravaginally
males inoculated intraurethrally (7).
etation of the antibody response fol-
intrarectal inoculation is complicated
fact that a number of animals was
fected in the eye and in the genital
One might have expected that in these
es an antibody response compatible
igher titers previously obtained fol-
infection at these sites would have
d. However, the antibody response
ave been influenced by the time
of infection as related to collection of

mary. Evidence for infection of male
male guinea pigs by the chlamydial
of guinea pig inclusion conjunctivitis
) following intrarectal inoculation was
ented by recovery of the agent from
swabs up to Day 23 and from seg-
of intestinal tract to Day 17. Anti-

body response to Gp-ic was demonstrated in
the serum by Day 21 although titers were
low as detected by indirect immunofluores-
cence. In a few instances scrapings of intes-
tinal tissue were found to contain cells with
Gp-ic inclusions when stained with Giemsa.
Gp-ic inclusions were also observed in ocu-
lar smears from males and females (8 posi-
tive/35 tested) as well as vaginal smears (2
positive/16 tested). These studies augment
the value of this animal model system for
the study of genital infections caused by
Chlamydia.

This paper is devoted to our friend Doctor Felix
Milgrom of the State University of New York at
Buffalo on the occasion of the thirtieth anniversary of
his research activities.

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Effects of Promethazine Hydrochloride on the Metabolism of Rabbit Alveolar Macrophages¹ (39553)

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Promethazine hydrochloride has been used clinically with impressive results to ameliorate the effects of erythroblastosis fetalis (1). Initial studies to define a mechanism of action demonstrated that administration of promethazine to experimental animals resulted in inhibition of both the primary and secondary immune response as well as delayed hypersensitivity (2). However, depression of circulating anti-Rh antibody titers has been noted in only some of the patients treated with the drug (J. P. Gusdon, Jr., unpublished data). The possibility that the drug was acting at the level of phagocytic cells was suggested by studies demonstrating that promethazine *in vitro* markedly inhibits oxidative events associated with phagocytosis by human polymorphonuclear leukocytes (3). It has been shown in animals that both the primary and secondary responses to red blood cell antigens are macrophage dependent (4, 5). We have recently demonstrated that fetal macrophages *in vitro* bind Rh-positive red blood cells which have been coated with anti-Rh antibody; this binding is inhibited *in vitro* by the addition of promethazine · HCl (6). The present report extends these studies by demonstrating that the drug inhibits macrophage metabolism *in vitro* in a fashion previously demonstrated for polymorphonuclear leukocytes (3). Further, administration of the drug *in vivo* to rabbits partially blocked the

cellular activation induced by heat-killed *Bacillus-Calmette-Guerin*.

Materials and methods. *In vitro* studies. Alveolar macrophages were collected from female New Zealand White Rabbits by the lung lavage technique of Myrvik *et al.* (7), 3-4 weeks after the animals were injected iv with a sonic suspension of heat-killed *Bacillus-Calmette-Guerin* (BCG) in Bayol F (100 µg in 0.10 ml). The heat-killed BCG was kindly supplied by Dr. Quentin Myrvik. Lungs were lavaged with 80 ml of isotonic saline followed by two washes with the same medium. Contaminating red blood cells were lysed for 20 sec in deionized water, and the suspension was brought to isotonicity with 3.5% saline. Differential counts were performed by phase microscopy in a white cell-counting chamber and the isolated cells were suspended in Dulbecco's phosphate-buffered saline (PBS) to a concentration of 5×10^6 macrophages per ml. Cell purity was routinely greater than 80%, with the bulk of the contamination consisting of nonphagocytic lymphocytes.

The oxidation of [1-¹⁴C]glucose was determined as previously described for neutrophils; all solutions were prepared in PBS (3). The reaction was routinely run in the presence of 1 mM cyanide to inhibit mitochondrial oxidation, which is significant in this cell type. Phagocytosis was initiated in appropriate flasks by the addition of 1.0 ml of heat-killed *Escherichia coli* B. The bacterial suspension was prepared in PBS and was standardized to give an absorbance of 1.00 at 525 nm on a Beckman DU spectrophotometer.

The kinetics of the phagocytic process were determined by measuring the uptake of radiolabeled *E. coli* B as previously described (8). The buffer employed was PBS containing a final concentration of 10% nor-

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bbit serum to provide a source of ns.

in vivo studies. In order to determine if the effects observed *in vitro* might be biologically significant, a series of *in vivo* studies was initiated. Animals were divided into four groups. Control: These animals received no treatment. BCG: These animals were injected iv with 100 μ g of killed BCG in Bayol F 3.5 weeks prior to sacrifice. Promethazine: Animals were treated daily with promethazine·HCl, 30 mg/kg body weight (im). BCG-Promethazine: Animals were first vaccinated with BCG and then injected with promethazine·HCl on a daily basis as above.

The concentration of promethazine in the *in vivo* studies is about 6 \times the maximal dose used in patient studies (1); it was selected because it was the dose employed in previous *in vivo* animal studies (2). We have not, however, determined serum levels of promethazine in rabbits, nor have serum levels following ingestion of the drug been determined, so direct comparison of the *in vivo* and *in vitro* studies is not possible. The results of animals in each group and physiological data on the groups of animals are presented in Table I. Vaccination of the animals with BCG caused a marked increase in weight of the lungs and a 10-fold increase in the number of cells obtained from the lungs compared with previous reports (9, 10). Simultaneous treatment with promethazine partially blocked this response, but the dif-

ferences are not impressive. This dose of promethazine (either alone or in vaccinated animals) appeared to cause some weight loss over the 3.5-week period of the experiment. Cells from all control animals were pooled, as were the cells from the promethazine-treated and the BCG-promethazine animals because of the low yield of cells per animal; the cells from the three BCG-treated animals were assayed separately.

The oxidation of [1- 14 C]glucose was determined on these cells as previously described. Serum was replaced in the incubation mixture by an equivalent amount of glucose solution (80 mg/dl). This was done to eliminate effects of different sera on the cells. Latex spherules (0.81 μ m) were employed as the challenge particle because they do not require opsonization for phagocytosis.

Results. Table II illustrates the effect of the *in vitro* addition of promethazine·HCl on the oxidation of [1- 14 C]glucose by BCG-induced rabbit alveolar macrophages. Resting cells exhibit an appreciable capacity to oxidize glucose via the hexose monophosphate shunt; this is stimulated two- to threefold by the initiation of phagocytosis, in substantial agreement with previously published reports (9, 10). The addition of promethazine to a final concentration of 0.033 mg/ml causes a profound inhibition of glucose oxidation under either resting or phagocytizing conditions. This inhibition is dose dependent; increasing the concentration of

TABLE I. ANIMAL DATA FOR *In vivo* PROMETHAZINE·HCl EXPERIMENT.^a

Treatment	Weight of start (kg)	Weight at end (kg)	Lung weight (g)	Packed cell volume
Control (8)	2.06 \pm 0.06	2.25 \pm 0.09	8.98 \pm 0.51	0.18 \pm 0.03
Promethazine (8)	2.07 \pm 0.11	1.65 \pm 0.08	7.58 \pm 0.40	0.10 \pm 0.02
Promethazine (6)	2.11 \pm 0.08	1.85 \pm 0.09	12.90 \pm 0.64	1.20 \pm 0.10
BCG (6)	2.02 \pm 0.19	2.22 \pm 0.14	16.30 \pm 1.44	1.83 \pm 0.44

Values represent the mean \pm SE for the number of animals given in parentheses.

TABLE II. EFFECT OF PROMETHAZINE·HCl ON OXIDATION OF [1- 14 C]GLUCOSE BY BCG-INDUCED RABBIT ALVEOLAR MACROPHAGES.^a

Description	Counts per minute in $^{14}\text{CO}_2$	
	Resting cells	Phagocytizing cells
Control	3204 \pm 78	7983 \pm 285
+ 0.033 mg/ml of promethazine·HCl	949 \pm 21	1284 \pm 60
+ 0.16 mg/ml of promethazine·HCl	408 \pm 4	393 \pm 10

Values represent the mean \pm SE for triplicate determinations.

promethazine to 0.16 mg/ml results in virtually complete inhibition of glucose oxidation. In experiments not shown, promethazine caused a similar inhibition of oxygen consumption by intact cells under both resting and phagocytizing conditions. These results cannot be attributed to an effect on cell viability since greater than 80% of the cells remained viable at the end of the incubation, as monitored by trypan blue exclusion.

This effect can be partially explained by an effect on ingestion as illustrated in Table III. Promethazine at a concentration of 0.16 mg/ml inhibits the uptake of radiolabeled bacteria at all time points examined. The inhibition of uptake, however, is not nearly as pronounced as the inhibition of cellular metabolism; also the effect on resting cells must be independent of any effects on particle uptake. Thus, the drug must exert a specific effect on macrophage oxidative metabolism which is independent of any effect on ingestion.

In an attempt to determine whether the effect of promethazine might be physiologically significant, we turned to *in vivo* experiments, as described in Materials and Methods. Results of these experiments are listed in Table IV. Vaccination of an animal with BCG results in marked activation of the alveolar macrophage, as indicated by the dramatic increase in [$1\text{-}^{14}\text{C}$]glucose oxidation. This activation is most apparent in resting cells, but is also observed in cells phagocytizing latex. The daily administration of promethazine to the BCG-treated animals causes a significant ($P < 0.001$)

inhibition of the cellular activation. This inhibition is observed under both resting and phagocytizing conditions.

Discussion. In a doctoral thesis in France, promethazine was first reported to be effective in ameliorating the effects of erythroblastosis in babies (Bierme Alie Enjalbert, Doctoral Thesis, Centre Regional de Transfusion Sanguine et d'Hematologie, Toulouse, France, 1967). Published clinical experiments conducted at this institution have generally supported this observation (1). To date, the effectiveness of promethazine hydrochloride in ameliorating the disease has been studied over the past 7 yr in more than 40 patients. It appears as though the fetal mortality has been reduced by at least 60% in this disease process (J. P. Gusdon, Jr., unpublished observations). More extensive clinical trials are currently underway with six other collaborating institutions.

The mechanism whereby the drug accomplishes this effect is uncertain, but promethazine has been demonstrated to cross the placenta (11, 12). It is likely that the effectiveness of this drug in ameliorating the effects of erythroblastosis is a function of its ability to impair the fetal reticuloendothelial cells (macrophages and/or lymphocytes) which are responsible for red cell lysis. In a previous study, we demonstrated that the *in vitro* addition of promethazine inhibited the ability of fetal macrophages to bind Rh-positive red blood cells (6); this has recently been demonstrated in the neonatal infant as well (13). The present study extends these observations by describing specific effects of promethazine hydrochloride on macrophage metabolism both *in vitro* and *in vivo*, thus, lending support to the concept that the drug acts at the level of the macrophage.

It seems possible that the primary effect of the drug might be at the level of the cell membrane, resulting both in decreased binding to opsonized red blood cells and to altered cellular metabolism.

Summary. The addition of promethazine hydrochloride to a suspension of rabbit alveolar macrophages *in vitro* results in an inhibition of cellular glucose oxidation under both resting and phagocytizing conditions as well as in an inhibition of phagocytosis of radiolabeled bacteria. The *in vivo*

TABLE III. EFFECT OF PROMETHAZINE·HCl ON PHAGOCYTOSIS OF *E. coli*- ^{14}C BY BCG-INDUCED RABBIT ALVEOLAR MACROPHAGES.^a

Time (min)	Cell-associated bacteria (cpm)	
	Control	Promethazine
0	936 \pm 99	846 \pm 7
5	5,987 \pm 585	3,140 \pm 241
10	14,152 \pm 610	8,344 \pm 263
15	18,023 \pm 471	12,279 \pm 676
20	21,644 \pm 128	12,257 \pm 276

^a Values represent the mean \pm SE for triplicate determinations. Promethazine was added, where indicated, to a final concentration of 0.16 mg/ml. Cells were challenged with 86,400 cpm of radiolabeled *E. coli* B.

TABLE IV. OXIDATION OF [1-¹⁴C]GLUCOSE BY ALVEOLAR MACROPHAGES OBTAINED FROM RABBITS UNDER VARIOUS EXPERIMENTAL CONDITIONS.^a

Treatment	Counts per minute in ¹⁴ CO ₂	
	Resting cells	Phagocytizing cells
Control (8)	4,563 ± 63	10,708 ± 87
Promethazine (8)	3,894 ± 40	6,714 ± 127
BCG-Promethazine (6)	10,250 ± 213	16,432 ± 322
BCG	22,399 ± 150	38,018 ± 805
BCG	31,513 ± 299	40,558 ± 231
BCG	21,834 ± 15	29,711 ± 1390

^a Values represent the mean ± SE of triplicate determinations. Cells were pooled from the number of animals indicated in parentheses; data for the three BCG-induced animals are given separately.

injection of the compound into rabbits partially suppresses the BCG-induced activation of the alveolar macrophages. These results suggest that the mechanism of action of promethazine hydrochloride in ameliorating the effects of erythroblastosis might lie, at least in part, in its ability to suppress the fetal macrophages which are responsible for red cell lysis.

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Tensile Properties of Tendon in Copper-Deficient Swine¹ (39554)

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Reduced tensile strength and elastic moduli of aorta (1) and aortic elastin (2) in copper-deficient swine have been related to the decrease of intermolecular crosslinks in elastin (3) due to a deficiency of a copper-containing enzyme, lysyl oxidase (4). Collagen is also a substrate for this enzyme and similar cross-linkages are accredited with the stabilization of collagen fibrils (5). The relative extent to which collagen as opposed to elastin cross-linkages contribute to the tensile properties of copper-deficient tissues has been difficult to determine (6). The present study was undertaken to compare the effect of copper deficiency upon the tensile properties of a purely collagenous tendon with its known effect upon the complex collagenous and elastic tissue of aorta.

Materials and methods. Seven crossbred pigs from two litters were reared from 2 days of age on a basal diet of evaporated cow's milk diluted with sulfide water, supplemented with iron (7). From age 2 weeks, three of the pigs were supplemented with 0.5 mg of copper/kg body weight per day as aqueous copper sulfate to serve as controls. The volume of packed red blood cells (VPRC) and serum copper levels (by atomic absorption spectrometry) were monitored weekly after age 1 month. All the pigs were sacrificed by exsanguination under pentobarbital anesthesia at ages 61-68 days when the VPRC and serum copper levels of the experimental group indicated severe deficiency (Table I). Segments of descending thoracic aorta and tails were stored at -70° until tested. Samples of tendon were fixed for electronmicroscopy in 3% glutaraldehyde in phosphate buffer, postfixed in 2% OsO_4 , dehydrated, stained with phosphotungstic acid in absolute ethanol, and

embedded in Durcupan ACM² for sectioning. Sections stained with uranyl acetate and lead citrate were examined with a Siemens Elmiskop IA.

Aortic rings, 2 mm wide, were stretched on the device described previously (1) in phosphate-buffered saline at pH 7.4 with simultaneous recording of load measured by a Statham transducer and extension at a constant rate of 5 mm/min. Tendons were stripped of their sheaths, gripped in the jaws of alligator clamps between filter papers, and stretched on the same apparatus. Calculations of elastic moduli and ultimate tensile strength were made from measurements of cross-sectional diameters and unstrained length with the ocular micrometer of a stereomicroscope. Ultimate tensile strength was defined as the force (grams) at the breaking point divided by cross-sectional area (square centimeters) at rest. Extensibility was defined as increment of length (millimeters) at the yield point divided by the initial extended length at zero force. Elastic modulus was defined by the formula $(\Delta F/A)/(\Delta L/L)$, in grams per square centimeter per 100% elongation, utilizing load (F), cross-sectional area (A), and initial length (L). Elastic recoil was determined by repeated elongation under 50-g increments of load up to 400 g, returning to the initial length between successive elongations. Stress-relaxation behavior was determined at successive 50-g increments of load by time tracings until relaxation was complete.

Results. Parameters of copper deficiency in the experimental group are given in Table I. The serum copper levels were about 5% of the controls. The aortic tensile strength and elastic modulus were less than 30% of the controls, indicating severe weakening of the aortic walls.

Tail tendons of the same deficient pigs failed to show any significant change in mechanical properties. Tensile strength, elastic modulus, and strain at breaking were the

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² Registered trademark of Fluka AG Chemische Fabrik, 9470 Buchs SG, Switzerland.

same as those of the controls at the 5% level of confidence (Table II). Elastic recoil (Fig. 1) was unchanged below the yield point. Decay of stress, due to relaxation at constant elongation, amounted to about 50% of the maximal stress in the linear phase of the stress-strain curve (Fig. 2) and was indistinguishable from the control.

The ultramicroscopic structure of the deficient tail tendons, in keeping with their mechanical properties, did not differ appreciably from the controls. The tendon proper contained no elastin (Fig. 3), although the sheaths, which were removed before stretching, contained small amounts.

Discussion. The syndrome of copper deficiency is characterized by spontaneous rupture of arteries (7, 8). Defects of vascular elastic membranes precede the ruptures (9). Loss of mechanical strength of whole aorta in copper-deficient swine (1) is accompanied by a commensurate loss of strength of the isolated aortic elastin (2). Repair of aortic elastin and restoration of mechanical strength follow administration of copper to deficient swine (10). The change in mechanical properties of elastin is correlated with a decrease in lysine-derived intermolecular cross-links (3) and their aldehyde intermediates (11). This has been attributed to deficiency of a copper-containing lysyl oxidase which oxidizes the ϵ -amino group of peptidyl lysine to the corresponding aldehyde (4).

Collagen is also a substrate for lysyl oxidase and analogous lysine-derived intermolecular cross-links occur in collagen (12). Decrease in cross-linkage of collagen in cop-

per deficiency is indicated by an increase in salt-soluble collagen in pig aorta (3) and chick Achilles tendon (13) and in acid-soluble collagen of chick bone (14). Alteration of the tensile properties of aortic collagen would be expected in copper deficiency but it has not been demonstrated because the physical structure of aortic collagen renders it unsuitable for stress-strain measurements (6). The structure of tail tendon fulfills the requirements which recommends its use for the purpose.

In view of the foregoing considerations, the failure of severe copper deficiency to affect the tensile properties of tail tendon is unexpected and warrants explanation. It suggests the possibility that tendon collagen cross-linkage may not be affected by copper deficiency, at least not to the extent that aortic elastin cross-linkage is affected. Previously reported work on chick bone and tendon makes this appear unlikely. Experiments have been undertaken to explore that possibility.

Meanwhile the alternative must be considered, namely, that the cross-links affected by copper deficiency may not be the limiting factor in the tensile properties of tendon that were tested, as they apparently are in elastin. While it is generally believed that these cross-links are responsible for the mechanical stabilization of collagen fibers, direct proof of this has not been found (15).

An important difference between the structure of elastic membranes and collagenous fibers may be a critical consideration in this problem. Elastin is a polymeric continuum and breakage of elastin laminae neces-

TABLE I. PARAMETERS OF COPPER DEFICIENCY.

Group	No. pigs	No. samples	Serum copper ($\mu\text{g}/100\text{ ml}$)	Aortic tensile strength (kg/cm^2)	Aortic elastic modulus ($\text{kg}/\text{cm}^2/100\%$ elongation)
Control	3	12	104.0 ± 4.0	11.30 ± 1.12	81.90 ± 15.37
Copper deficient	4	16	5.3 ± 0.7^a	3.36 ± 0.58^a	21.85 ± 5.73^a

^a $P < 0.001$.

TABLE II. TENSILE PROPERTIES OF PIG TAIL TENDON.

Group	No. pigs	No. tendons	Tensile strength (kg/cm^2)	Elastic modulus ($\text{kg}/\text{cm}^2/100\%$ elongation)	Strain at breaking point (% elongation)
Control	3	18	352 ± 57	1321 ± 133	36.0 ± 2.0
Copper deficient	4	27	327 ± 83^a	1338 ± 82^a	36.8 ± 2.0^a

^a $P > 0.05$.

sarily involves rupture of covalent bonds. Collagen consists of discontinuous subunits (fibrils) which may separate from one another by slippage, rather than by breakage (16). The role of weak bonds may be predominant in determining mechanical behavior of the highly ordered collagen molecules within the fibrils (17). The elastic recoil and stress-relaxation behavior indicate that these bonds are not affected by copper deficiency.

Summary. Young pigs were rendered

copper deficient as demonstrated by serum copper levels and aortic tensile properties. Tail tendons of the same pigs had normal ultimate tensile strength, elastic modulus, elastic recoil, and stress-relaxation compared to controls. These results suggest that lysine-derived cross-links of insoluble collagen of tendon may not be decreased in copper deficiency, as are the analogous elastin cross-links, or that such cross-linkages may not be the limiting factor in determining tensile strength of collagen, as they appear to be in elastin.

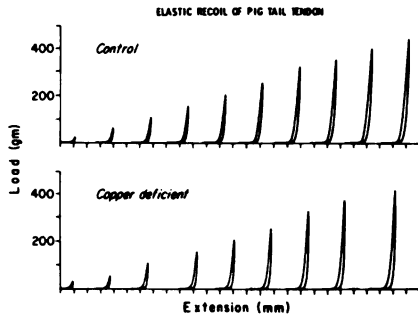


FIG. 1. Elastic recoil of pig tail tendon repeatedly extended at rate of 5 mm/min by increasing loads.

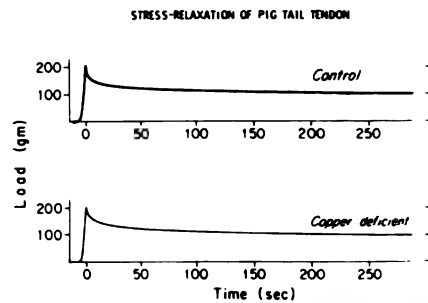


FIG. 2. Stress-relaxation of pig tail tendon maintained at constant extension.



FIG. 3. Electronmicrograph of control pig tail tendon. Small elastic fibers (arrow) are confined to the sheath (S). Collagenous fibrils of the tendon (T), fixed under extension by 50-g load, appear to have a low-pitched helical arrangement.

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Levels and Significance of Erythrocyte Purine Enzymes in Hyperuricemia (39555)

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The pathogenesis of hyperuricemia and gout is still largely undefined (1, 2) but it must depend ultimately upon an alteration in the synthesis and/or disposal of uric acid. Several enzymatic abnormalities have been related to an increased uric acid production. These include a deficiency in hypoxanthine-guanine phosphoribosyltransferase, a deficiency of glucose-6-phosphatase, increased activity of phosphoribosylpyrophosphate synthetase, increased activity of glutathione reductase, and increased activity of xanthine oxidase (1).

Recent studies (3, 4) on purine interconversion and salvage in mammalian cells reported an inadequate activity of purine salvage and interconverting enzymes led to excessive production and excretion of purines. These investigators introduced the concept of the "adenosine cycle" whose functioning at a high rate was predicted to lead to an increased concentration of uric acid precursors such as hypoxanthine and xanthine. The activity of the cycle was regulated by adenosine kinase (3, 4). The presence of most of the enzymes of the adenosine cycle, adenosine deaminase (5-9), adenosine kinase (5-7), hypoxanthine-guanine phosphoribosyltransferase (10-15), and 5'-nucleotidase (16), has been reported in the human erythrocyte.

In view of the possible association between the activity of the adenosine cycle and the rate of purine synthesis, this report describes the activities of the following enzymes: adenosine kinase (EC 2.7.1.20), adenosine deaminase (EC 3.5.4.4), hypoxanthine phosphoribosyltransferase (EC 2.4.2.8), adenine phosphoribosyltransferase (EC 2.4.2.7), and 5'-nucleotidase (EC 3.1.3.5) in erythrocytes from young adults who were normouricemic, hyperuricemic, or had a family history of gout. The activities of these enzymes were correlated with plasma uric acid concentrations to evaluate whether inappropriate activities of purine

salvage and interconverting enzymes in the erythrocyte might be associated with hyperuricemia and the development of clinical gout.

Materials and methods. Blood samples (10 ml) were collected in EDTA from 1174 University of California, Davis, students during their entering physical examination who willingly consented to participate in a study assessing the factors which may predispose them to premature coronary heart disease. The plasma was separated from the red blood cells by centrifugation at 2000g.

Erythrocytes from hyperuricemic (plasma uric acid >7 mg/100 ml), normouricemic (plasma uric acid <7 mg/100 ml), and those with a family history of gout (response to a questionnaire) were washed three times with physiologically buffered saline at 4° and the washed erythrocytes were again isolated by centrifugation. At each washing the buffy layer containing leukocytes was discarded with the washing. The washed erythrocytes were sonicated for 30 sec and centrifuged at 17,000g for 20 min at 4°. Two-and-one-half milliliters of 0.15 M phosphate buffer, pH 6.8, was added to 0.5-ml aliquots of the supernatant from sonicated erythrocytes and these extracts were stored at -90° until all the purine enzyme assays were performed. All enzyme assays were completed within 4 months after the samples were collected.

Plasma uric acid was determined by an enzymatic spectrophotometric method (17) and the protein content of the erythrocyte extracts by the method of Munro and Fleck (18). The protein concentration in the extracts was approximately 50 mg/ml.

Purine enzyme activities were measured as described by Shenoy and Clifford (19) and the methods are briefly summarized here. The activity of 5'-nucleotidase in hemolysates was measured by the production of adenosine, inosine, and hypoxanthine from [U-¹⁴C]AMP; inosine and hypoxanthine

from [U-¹⁴C]IMP; and guanosine and guanine from [U-¹⁴C]GMP. The reaction mixture contained three different final concentrations of AMP, GMP, or IMP: 0.0125 mM (sp act, 40 mCi/mmol), 0.0625 mM (sp act, 8 mCi/mmol), and 0.15 mM (sp act, 3.35 mCi/mmol); three different concentrations of MgCl₂: 0, 40, and 80 mM; phosphate (50 mM) or Tris-maleate buffer (100 mM, pH 6.8), and 5–30 μ l of erythrocyte extract in a total volume of 200 μ l (made up with deionized water). The reaction mixture was incubated for 10 min at 37°. The reaction was stopped with 15 μ l of ice-cold 70% HClO₄ and allowed to stand in ice for 15 min before being neutralized with 30 μ l of 7 N KOH. The mixture was centrifuged for 10 min (3000g at 4°) and 10 μ l of the supernatant was spotted on cellulose chromatographic sheets with appropriate standards. The plate was chromatographed (ascending) using deionized water as the developing solvent. Then the reactants and products were located under ultraviolet light, transferred to a vial containing 10 ml of scintillation fluid, and the radioactivity was measured.

Adenosine kinase, hypoxanthine phosphoribosyltransferase, and adenine phosphoribosyltransferase were assayed by measuring the amount of radioactive purine base or nucleoside incorporated into the product nucleotides which were isolated as lanthanum salts after precipitation with 0.5 M LaCl₃. The final reaction mixture for adenosine kinase contained 0.05 mM adenosine (sp act, 10 mCi/mmol), 50 mM phosphate buffer (pH 6.8), 2.5 mM ATP, 0.25 mM MgCl₂, and 10 μ l of erythrocyte extract (approximately 50 mg of protein per ml), in a total volume of 200 μ l (made up with deionized water).

The final reaction mixture for hypoxanthine phosphoribosyltransferase and adenine phosphoribosyltransferase contained 0.45 mM hypoxanthine or adenine (sp act, 1.1 mCi/mmol), 1 mM phosphoribosyl-1-pyrophosphate (dimagnesium salt), 0.625 mM MgCl₂, 50 mM phosphate buffer (pH 6.8), and 10 μ l of erythrocyte extract, in a total volume of 200 μ l (made up with deionized water). A reaction time of 10 min was used for these enzyme assays.

Adenosine deaminase was assayed by iso-

lating and measuring inosine, hypoxanthine, and IMP formed from labeled adenosine. The final reaction mixture contained 0.1 mM adenosine (sp act, 5 mCi/mmol), 50 mM phosphate buffer (pH 6.8), and 10 μ l of erythrocyte extract in a total volume of 200 μ l (made up with deionized water). After incubation for 8 min at 37°, the reaction was terminated with 15 μ l of 70% HClO₄, giving a final concentration of 1 N in the reaction mixture. The mixture was then heated for 1 hr in a boiling water bath in order to transform nucleotides and nucleosides to purine bases. The acid mixture was neutralized to pH 7.0 with 30 μ l of 7 N KOH, centrifuged at 3000g, and hypoxanthine was separated from adenine by thin-layer chromatography (ascending) using water as the developing solvent. The adenosine deaminase activity was determined from the radioactivity recovered in hypoxanthine. The accuracy of the procedure was checked by measuring the radioactivity remaining in adenosine or recovered in inosine, hypoxanthine, and IMP (without acid-heating) using the two-dimensional thin-layer chromatography method described by Henderson, *et al.* (20). Chemicals used in the present study were as we have described previously (19).

Results. For the population from which subjects were selected for the present study, a plasma uric acid level of 7.0 mg/100 ml was chosen as the upper limit of the normal range, and individuals with levels above this limit were defined as hyperuricemic. Using this definition, 4.1% of the total population were hyperuricemic and 7.4% had a family history of gout. Hyperuricemia occurred in 14.6% of those with a family history of gout.

From the total population of 1174 subjects (742 males, 432 females, mean age 23 \pm 5 years), 99 were selected for erythrocyte enzyme measurements. The selection was based upon plasma uric acid values and presence of a family history of gout. The distribution of the population into normo-uricemic, hyperuricemic, and family history of gout groups, and the purine enzyme activities are presented in Table I. The data demonstrate an absence of 5'-nucleotidase, a lack of striking differences in enzyme activities among the groups except for HPRT

which was lower (14 and 17%) in the hyperuricemic and family history of gout groups, respectively, compared with the normouricemic group and APRT which was higher (15%) in those with a family history of gout.

When the ratio APRT/HPRT was calculated, it was elevated by 19 and 36% in the hyperuricemic and subjects with a family history of gout, respectively, compared with the normouricemics. Sixty-six percent (4/6) of the hyperuricemic subjects who also had a family history of gout had an elevated APRT/HPRT ratio when compared with normouricemic subjects without a family history of gout. Only 33% (2/6) of the hyperuricemic subjects who also had a family history of gout had an elevated APRT/HPRT ratio when compared with normouricemic subjects with a family history of gout.

The mean APRT/HPRT ratio for the six hyperuricemic subjects with a family history of gout did not differ significantly from that of the normouricemic subjects in this group (Table II). Plasma uric acid levels and erythrocyte enzyme activities did not differ between males and females within each group.

All active enzyme assays resulted in a linear formation of product within 8- to 10-min assay periods and a linear product formation at different enzyme concentrations.

Discussion. The purpose of this investigation was to correlate the activity of purine enzymes and plasma uric acid in an attempt to evaluate the hypothesis that inappropriate patterns of activities of purine salvage and interconverting enzymes might be associated with hyperuricemia and the development of clinical gout.

TABLE I. ERYTHROCYTE PURINE ENZYME ACTIVITIES AND PLASMA URIC ACID LEVELS IN YOUNG ADULTS WHO WERE NORMOURICEMIC, HYPERURICEMIC, OR HAD A FAMILY HISTORY OF GOUT.

	Control	Hyperuricemic	Family history of gout
Number of subjects	27	31	41
Male subjects	21	29	23
Female subjects	6	2	18
Plasma uric acid (mg/100 ml)	4.9 ^a ± 1.1	8.1 ± 0.9 ^b	5.1 ± 1.8
Adenosine deaminase ^c	29.9 ± 13.7	31.4 ± 12.9	26.5 ± 8.8
Adenine phosphoribosyltransferase ^c	5.1 ± 1.1	5.5 ± 1.0	5.9 ± 1.1 ^d
Hypoxanthine phosphoribosyltransferase ^c	89.4 ± 15.2	79.3 ± 12.3 ^b	75.9 ± 17.2 ^b
Adenosine kinase ^c	11.2 ± 1.6	11.9 ± 1.8	11.5 ± 1.7
5'-Nucleotidase ^c	None	None	None
APRT/HPRT × 10 ³	59 ± 16	70 ± 16 ^b	80 ± 19 ^b

^a Values are means ± SD.

^b Horizontal values different from control ($P < 0.001$).

^c Enzyme activities are micromoles of product produced per gram of protein per hour. The products were adenosine deaminase = inosine + hypoxanthine; adenine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase, and adenosine kinase = LaCl₃-precipitated nucleotides; 5'-nucleotidase = adenosine + inosine + hypoxanthine or guanosine + guanine or inosine + hypoxanthine.

^d Horizontal values different from control ($P < 0.025$).

TABLE II. APRT/HPRT RATIO^a IN HYPERURICEMIC SUBJECTS WITH A FAMILY HISTORY OF GOUT.

Subject no.	Plasma uric acid (mg/100 ml)	APRT (μmol of product/g of protein/hr)	HPRT (μmol of product/g of protein/hr)	APRT/HPRT × 10 ³
0112	7.6	6.43	72.7	88
1947	9.7	5.16	76.9	67
1846	8.0	6.19	62.5	99
1668	9.7	5.16	76.9	67
1730	7.3	5.15	88.5	58
1036	7.9	4.13	85.1	48
Control ^b	4.5 ± 1.2	5.95 ± 1.09	75.7 ± 18.4	82 ± 19

^a Adenine phosphoribosyltransferase to hypoxanthine phosphoribosyltransferase ratio.

^b Mean of the 35 remaining normouricemic subjects with a family history of gout.

basis of the selection criteria used, subjects were hyperuricemic (mean uric acid 8.0 mg/100 ml). The normuricemic (controls) and the group with a history of gout had similar mean uric acid levels (Table I) which were in the range expected for young adults (2, 21, 22). This was somewhat surprising since in gout, a genetically linked condition having as a first-step hyperuricemia, a somewhat higher plasma uric acid in this group than in the controls might be expected. Since this was not the case, one might speculate that this group was composed of normouricemic individuals with a possibility of latent hyperuricemia and

variations in adenine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase activities in young adults with hyperuricemia or a family history of gout. This type of subject for the first time is represented by the first observations already made on Lesch-Nyhan and gouty patients (23). Although the biological significance of the above APRT/HPRT ratio is not yet clear, a comparison of the hypoxanthine phosphoribosyltransferase activity in subjects with low hypoxanthine phosphoribosyltransferase activity might reveal enzyme stabilization by increased hypoxanthine phosphoribosylpyrophosphate levels (24). The APRT/HPRT ratio which was elevated in subjects of high risk of gout [hyperuricemic or family history of gout (21)] could, if confirmed in a larger population of young adults, represent a diagnostic indicator of latent gout. This idea is supported by the data presented in Table II. It is especially noteworthy that the highest ratio of APRT/HPRT occurred in normouricemic subjects with a family history of gout.

Although the lack of correlation between erythrocyte hypoxanthine phosphoribosyltransferase and adenine phosphoribosyltransferase activity and serum uric acid has been reported previously (23), the lack of correlation between plasma uric acid and erythrocyte adenosine deaminase and adenine phosphoribosyltransferase activities has not been reported previously.

The low activity of erythrocyte 5'-nucleotidase in this study, together with the lack of a functional purine nucleotide system for the conversion of

IMP to AMP in the human erythrocyte (25), further suggests that the adenosine cycle (3) is incomplete in the human erythrocyte. An incomplete adenosine cycle probably explains the lack of correlation between the activity of purine enzymes and plasma uric acid levels, and suggests the need for utilizing other tissues, in addition to the erythrocyte, to search for this correlation. The higher ratio of APRT/HPRT in erythrocytes from subjects of high risk of gout could, if confirmed in a larger population of young adults, be of diagnostic value in preclinical gout.

Summary. To examine possible relationships among purine enzyme patterns in hyperuricemia and gout, purine enzyme activities were measured in erythrocytes from young adults who were either normouricemic, hyperuricemic, or had a family history of gout. The purine enzymes whose activities were measured included adenine phosphoribosyltransferase (APRT), hypoxanthine phosphoribosyltransferase (HPRT), adenosine deaminase (AD), adenosine kinase (AK), and 5'-nucleotidase. AD and AK activities were not different among the normouricemic, hyperuricemic, or family history of gout groups. The activity of APRT was significantly higher in the group with a family history of gout compared with the normouricemics, and the activity of HPRT was significantly lower in the hyperuricemic and family history of gout groups compared with the normouricemics. The ratio of APRT/HPRT was significantly greater in 66% of those subjects who had a family history of gout and were also hyperuricemic compared with normouricemics without a family history of gout. No measurable 5'-nucleotidase was found in human erythrocyte hemolysates. The lack of correlation between the activities of AD and AK in erythrocytes and plasma uric acid levels may be due to the lack of 5'-nucleotidase in this tissue since in the absence of 5'-nucleotidase the erythrocyte has a minimal capacity to break down nucleotides.

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Increased E-Rosette Formation following Streptolysin-O Treatment¹ (39556)

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Streptolysin-O (SO) is known to be cytotoxic to a number of cell types (1-5). Cell death at high SO doses (1, 2) and impaired cell function at lower doses (3-5) have been reported. Andersen and Cone (5) have shown that lymphocyte blast transformation, as measured by [³H]thymidine uptake, can be blocked by pretreating lymphocytes with sublytic doses of SO. Since SO has the potential of altering immunologic responses important in streptococcal infection, we further investigated the action of SO on human lymphocytes using the E-rosette (erythrocyte, anti-complement) cell rosette techniques.

Materials and methods. *Streptolysin-O.* SO was produced by Todd-Hewitt broth cultures of Richards strain, group A type 3 *Streptococcus pyogenes*, and isolated using precipitation and Sephadex G100 chromatography as described by Van Epps and Andersen (6). A hemolytic unit (HU) was defined as the minimal amount of SO that in an equal volume of a 2% sheep red cell (SRBC) suspension.

Lymphocyte isolation. Using the Ficoll-graue density gradient technique as described by English and Andersen (7), lymphocyte-rich fractions were isolated from normal human blood. The cells were washed three times with saline, counted in a cytometer, and adjusted to a concentration of 10⁷ cells per ml.

Rosettes. SRBCs were washed three times in phosphate-buffered saline (PBS), pH 7.2, and suspended to give a final SRBC concentration of 2% in 10% fetal calf serum in essential media. Equal volumes of SRBCs and lymphocytes (untreated or treated as described below) were incubated for 30 min at room temperature. The cells

were centrifuged at 200g for 5 min and incubated overnight at 4° without disturbing the cell button. After gentle suspension of the cells, 1 drop of 0.5% toluidine blue-O was added. The cells were placed in a hemocytometer, kept at room temperature for 5 min, and examined by light microscopy. Two hundred lymphocytes were examined for each test, and those with three or more attached SRBCs were counted as rosettes. The percentage of lymphocytes forming rosettes was determined.

EAC-Rosettes. An equal volume of rabbit anti-SRBC diluted 1:300 was added to SRBCs that had been washed four times with PBS. After incubation at 4° for 1 hr, the cells were washed twice with PBS to remove free hemoglobin. These cells can be stored for 1 week in the refrigerator. Before use, 9.6 ml of a 5% solution of the EAC cells were mixed with 0.4 ml of fresh normal human serum and incubated for 1 hr at 37°. These EAC cells were washed with PBS till the free hemoglobin was gone, and then adjusted to a 2% suspension with PBS. Equal volumes of EAC cells and treated or untreated lymphocytes were incubated at room temperature for 30 min while tumbling end-over-end at 30 rpm. Incubation was continued at 4° for 30 min with tumbling. Prior to counting rosettes, the preparation was mixed vigorously on a vortex mixer. The counting procedure was the same as that described for the E-rosettes.

Treatment protocol. Isolated human lymphocytes were used in all experiments. The cells were treated with hemolytically active SO or with SO inactivated by treatment with anti-SO serum, cholesterol, heat (56°C, 30 min), or oxygen. In some cases, the lymphocytes were treated first with SO, then with cholesterol or anti-SO serum to determine the reversibility of the SO effect. Treatment periods were always 30 min and incubation temperature was 37°. Following treatment,

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the lymphocytes were washed twice with PBS before the rosette assays were performed. All experiments were done in duplicate and every experiment was repeated at least once.

Results. When low levels of hemolytically active SO ($SO \leq 2$ HU) were incubated with isolated lymphocytes, the percentage of T-cells, as determined by the E-rosette technique, consistently decreased (Fig. 1). This effect was dose related, and very few E-rosettes could be found when 2 HU of SO was used. Cholesterol, which inhibits the hemolytic activity of SO, was found to block the effect of SO on lymphocytes, but could not reverse the effect after SO had been incubated with the lymphocytes (Table I). The decrease in E-rosette levels was not due to lysis of lymphocytes by SO since there were no differences in the number of lymphocytes before and after SO treatment when 2 HU or less was used. There was no significant change in the percentage of EAC-rosettes following SO treatment.

Table II demonstrates that the effect of SO on E-rosette formation could not be reversed by the anti-SO serum after the SO had been incubated first with the lymphocytes. The SO effect could be blocked, however, when the SO was preincubated with the anti-SO serum. Other factors which will

inactivate the hemolytic ability of SO, namely, heat (56° , 30 min) and oxygen were effective in blocking the action of SO on the formation of E-rosettes.

Discussion. Small amounts of SO were capable of markedly reducing the number of E-rosettes found in isolated human blood lymphocyte preparations. The ability of agents which can block the hemolytic action of SO to interfere with the E-rosette blocking of SO suggests that the mechanism causing hemolysis is related to the one which blocks E-rosette formation. The effect of SO on E-rosette formation was not due to lysis of lymphocytes since lysis fails to occur at the levels of SO used in these experiments. A previous study of lymphocyte viability using the Evans blue dye exclusion method has shown that 1 HU of SO will not alter cell viability (5). No change was observed in the number of EAC-rosettes following SO treatment.

The effect of SO on E-rosettes was not due to the direct action of SO on the SRBCs, since SO was removed by washing the lymphocytes prior to the assay. Possible action of small amounts of residual SO on the SRBC-rosette was discounted by data shown in Table II. The SO effect on E-rosette formation could not be reversed by adding anti-SO serum following SO treatment. Since the anti-SO serum was added prior to the SRBCs, the antiserum would have neutralized any residual SO activity and protected the SRBCs from the effect of SO. In spite of this, the percentage of E-rosettes following SO treatment was markedly reduced compared to normal.

The mechanism of SO action on E-lymphocytes is unknown. Since other studies have indicated that its primary action is on cell membranes, it seems likely that SO is altering the SRBC receptor site of T-lymphocytes. The failure of cholesterol and anti-SO serum to reverse the effect suggests that membrane structure has been irreversibly altered.

An earlier study from this laboratory (5) has shown that T-lymphocyte blast transformation by PHA can be blocked by pretreatment of lymphocytes with SO. This action of SO may be due to the same or a similar membrane effect that has been postulated

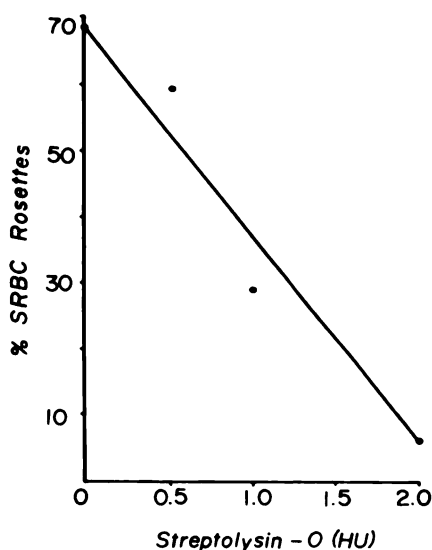


Fig. 1. The effect of varying concentrations of streptolysin-O on E-rosette (SRBC-rosette) formation.

TABLE I. EFFECTS OF STREPTOLYSIN-O AND CHOLESTEROL ON E- AND EAC-ROSETTES.

Lymphocyte treatment	E-Rosettes (%)		EAC-Rosettes (%)	
	Duplicates	Mean	Duplicates	Mean
None	67,70	68	15,15	15
SO (1 HU)	27,30	28	13,13	13
Cholesterol ^a	62,64	63	N.D. ^b	
SO (1 HU) → cholesterol ^c	33,36	34	N.D.	
SO (1 HU)/cholesterol ^d	62,66	64	N.D.	

^a Lymphocytes treated with a saturated solution of cholesterol for 30 min at 37°.

^b N.D., not done.

^c Lymphocytes treated first with SO (1 HU) for 30 min at 37°, then incubated with an equal volume of a saturated solution of cholesterol for 30 min at 37°.

^d SO (1 HU) mixed with a saturated solution of cholesterol prior to treatment of lymphocytes for 30 min at 37°.

TABLE II. EFFECTS OF ANTI-STREPTOLYSIN-O SERUM ON THE STREPTOLYSIN-O SUPPRESSION OF E-ROSETTES.

Lymphocyte treatment	E-Rosettes (%)	
	Duplicates	Mean
None	60,76	68
SO (2 HU)	10,14	12
SO (2 HU) → ASO ^a	16,17	16
ASO	64,68	66
SO (2 HU)/ASO ^b	67,73	70

^a Lymphocytes treated first with SO (2 HU) for 30 min at 37°, then incubated for 30 min at 37° with an equal volume of human serum containing anti-SO activity (333 Todd units).

^b SO (2 HU) mixed with human anti-SO serum (333 Todd units) prior to treatment of lymphocytes for 30 min at 37°.

or the E-rosette inhibition. These observations suggest that SO may act *in vivo* to alter the T-lymphocyte function of the host.

Summary. Doses of SO which did not cause lymphocyte lysis or reduced viability

(≤ 2 HU), decreased E-rosette formation. SO, treated with agents which block hemolytic activity (heat, oxygen, cholesterol, and anti-SO serum), did not affect E-rosette formation. Cholesterol and anti-SO serum were not able to reverse the E-rosette-inhibiting action of SO. EAC-rosettes were not affected by SO.

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Effects of Bradykinin on Rat Lymphocytes (39557)

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Vasoactive peptides (bradykinin and related kinins) are released in injured tissues from protein substrates by a variety of proteolytic enzymes. Kinins are considered to mediate inflammatory processes due to their ability to induce vasodilation, increased blood vessel permeability, and pain. Recently to the list of these well-recognized physiological effects of bradykinin, a new one has been added, namely, stimulation of mitotic activity of rat thymocytes, possibly by a cAMP-mediated reaction (1, 2). This, if confirmed, for peripheral lymphatic cells would enlarge considerably the role of bradykinin in mediation of tissue response to injury, since the stimulated lymphatic cell may be a source of multiplicity of factors affecting the inflammatory reaction and wound healing.

The aim of the present work is to investigate the effects of bradykinin on lymphocytes from rat peripheral lymph nodes.

Materials and methods. Female Wistar rats, 3- to 4-months old, were bled under ether anesthesia, and then axillary, submandibular, inguinal, and mesenteric lymph nodes were removed. Lymphocyte suspensions were prepared by dissection of lymph nodes with preparative needles and placed in the medium 199 (Biomed, Lublin, Poland). Bradykinin (Koch Light Lab., Colnbrook, England) was dissolved in the medium 199. [^3H]Thymidine, 2 Ci/mole, was purchased from UVVVR, Prague, Czechoslovakia. [^3H]uridine, 20-30 Ci/mole, [^{14}C]leucine, 62 mCi/mole, and NCS tissue solubilizer were the products of Radiobiological Centre, Amersham, England. Lymphocytes (10^7) were cultivated in medium 199, supplemented with 20% (vol/vol) of calf serum and 0.03% L-glutamine, in final volume samples of 2 ml, at 37° for various times up to 96 hr. Bradykinin was added at the beginning of cultivation.

Incorporation of [^3H]thymidine and

[^3H]uridine was determined as described by Bauscher *et al.* (3). Washing of TCA precipitates with methanol was omitted. [^3H]Thymidine (2 μCi per culture) or [^3H]uridine (1 μCi per culture) was added 18 or 24 hr before the cell harvest, respectively.

Incorporation of [^{14}C]leucine (1 μCi per culture) added 4 hr before cell harvest was determined as described by Kay *et al.* (4). TCA precipitates were separated by centrifugation at 1000 g for 15 min (instead of separation on Millipore filters) and dissolved in 0.5 ml of NCS tissue solubilizer. Radioactivity measurements were carried out using POP-POPOP-toluene liquid scintillator and Mark II Nuclear Chicago counter.

Viability of lymphocytes was estimated by 0.2% trypan blue exclusion test. Bradykinin concentrations used did not affect significantly cell numbers and viability during the 96-hr cultivation, as compared with the appropriate controls.

Results. Effects of bradykinin on incorporation of [^3H]thymidine, [^3H]uridine, and [^{14}C]leucine are summarized in Tables I, II, and III, respectively. The data represent mean values from 10 to 15 determinations with the standard deviations. Incorporation of the precursors was markedly enhanced by all concentrations of bradykinin employed. At its lowest level, 1.0 μM , the increments over the control values were moderate (16-43%) but significant ($P < 0.05$) after 24 hr of culture, and they increased rapidly to 200-300% of control values after 72 hr. No regular dependence of the incorporation on bradykinin concentration was observed. However, the values found at 10- μM bradykinin concentration tend to exceed those found at 1 and 5 μM of bradykinin.

The stimulating activity of all applied bradykinin concentrations was evident as early as after 24 hr. The maximal increase in

TABLE I. EFFECT OF BRADYKININ ON [^3H]THYMIDINE INCORPORATION INTO LYMPHOCYTES ($\text{CPM} \times 10^{-3}$ PER SAMPLE).

Bradykinin (μM)	Cultivation time (hr)			
	24	48 ($\text{cpm} \times 10^{-3}$ per sample)	72	96
0	3.2 ± 0.4	2.6 ± 0.3	1.6 ± 0.4	1.4 ± 0.2
1.0	4.2 ± 0.2	4.8 ± 0.3	5.4 ± 0.9	3.3 ± 0.2
5.0	4.0 ± 0.2	4.6 ± 0.4	5.0 ± 0.3	3.1 ± 0.4
10.0	4.5 ± 0.2	5.7 ± 0.7	8.0 ± 1.0	5.1 ± 0.9
20.0	4.2 ± 0.3	4.9 ± 0.4	5.3 ± 0.6	2.4 ± 0.5

TABLE II. EFFECTS OF BRADYKININ ON SPONTANEOUS INCORPORATION OF [^3H]URIDINE INTO LYMPHOCYTES ($\text{CPM} \times 10^{-3}$ PER SAMPLE).

Bradykinin (μM)	Cultivation time (hr)			
	24	48 ($\text{cpm} \times 10^{-3}$ per sample)	72	96
0	56.3 ± 3.3	29.1 ± 2.9	23.2 ± 3.3	18.8 ± 4.5
1.0	65.2 ± 2.8	68.5 ± 5.5	53.3 ± 6.8	49.7 ± 7.0
5.0	72.1 ± 2.5	79.7 ± 6.8	51.1 ± 3.5	52.6 ± 4.1
10.0	89.3 ± 4.9	101.7 ± 9.3	74.3 ± 7.3	62.3 ± 5.0
20.0	89.7 ± 3.8	87.8 ± 7.1	56.6 ± 3.2	44.5 ± 2.7

TABLE III. EFFECTS OF BRADYKININ ON SPONTANEOUS INCORPORATION OF [^{14}C]LEUCINE INTO LYMPHOCYTES ($\text{CPM} \times 10^{-3}$ PER SAMPLE).

Bradykinin (μM)	Cultivation time (hr)			
	24	48 ($\text{cpm} \times 10^{-3}$ per sample)	72	96
0	1.6 ± 0.1	0.8 ± 0.3	0.7 ± 0.3	0.6 ± 0.3
1.0	2.3 ± 0.2	2.5 ± 0.4	2.1 ± 0.4	1.5 ± 0.3
5.0	2.1 ± 0.2	2.4 ± 0.4	1.9 ± 0.2	1.6 ± 0.3
10.0	2.4 ± 0.3	3.0 ± 0.6	2.1 ± 0.2	1.6 ± 0.2
20.0	2.5 ± 0.3	2.7 ± 0.5	2.3 ± 0.3	1.8 ± 0.3

[^3H]thymidine incorporation was observed after 72 hr while maximal effects of bradykinin on incorporation of [^3H]uridine and [^{14}C]leucine were detected 24 hr earlier, i.e., after 48-hr cultivation.

Discussion. A number of observations reported recently indicates that low-molecular weight regulatory substances, such as catecholamines, prostaglandins, histamine (5), as well as peptide hormones, influence the proliferation and function of lymphatic cells. Some of the peptide hormones, such as growth hormone (6), parathormone (7), and vasopressin (8), were investigated previously only in respect to their stimulatory effect on proliferation of thymocytes.

On the contrary, chorionic gonadotropin and chorionic somatomammotropin were found to inhibit the blastogenic response of human lymphocyte. These effects of placen-

tal hormones were postulated to be of a probable significance for maternal lymphocyte immunocompetence and fetus survival (9-12).

Information about the effects of bradykinin and related kinins on lymphatic tissue is scarce. Perris and Whitfield (1) and Whitfield *et al.* (2) showed that bradykinin stimulates incorporation of [^3H]thymidine and mitotic activity in rat thymocytes *in vitro*. The data reported in this paper reveal a strong effect of bradykinin on peripheral lymph node cells of rat. Similarly to the above-mentioned authors, we have observed a pronounced increase of [^3H]thymidine incorporation, and have found that it was accompanied by an increased incorporation of [^3H]uridine and [^{14}C]leucine. As these effects did not seem to be concentration dependent, the lowest brady-

kinin concentration employed, 1.0 μM , is perhaps sufficient to generate the maximum response resulting in a plateau at all other concentrations. However, shape of the dose-response curve could have been affected by the presence of serum in the culture medium, since presumably it contained active kininases. The addition of serum was, of course, necessary to maintain viability of the lymphocytes during the 96-hr cultivation. This would tend to equalize responses at various concentration levels, the notion supported by a rather consistent occurrence of higher precursor incorporation at a high (10 μM) bradykinin concentration. The stimulating effect of bradykinin below 1.0 μM has not been as yet, to our best knowledge, investigated in lymphatic cells. It now becomes important to estimate the lowest effective bradykinin concentration in order to assess the relevance of the observed phenomena for physiological processes.

The time course of the incorporation differed with various precursors tested. Peak values for [^3H]uridine and [^{14}C]leucine incorporation occurred after 48-hr incubation and were followed by [^3H]thymidine maximum at 72 hr. This sequence of events is considered to characterize the response of lymphocytes to specific antigens, as well as to nonspecific mitogens (13). As the lymphocytes used in the present studies were cultivated in the presence of heterologous calf serum, the question arises whether the bradykinin-enhanced stimulation of protein and nucleic acid synthesis is connected with, or is independent of activation of, the lymphocyte immunological functions. It seems likely that the effects observed in this work are caused by the direct action of bradykinin, since Perris and Whitfield (1) and Whitfield *et al.* (2) have shown that the mitotic

activity of thymocytes was stimulated by bradykinin in a serum-free medium.

Summary. Bradykinin stimulates incorporation of [^{14}C]leucine, [^3H]uridine, and [^3H]thymidine into cultured lymphocytes of rat lymph nodes. Increase in [^{14}C]leucine and [^3H]uridine incorporation precedes that of [^3H]thymidine.

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Acetylcholine and Gastric Secretion¹ (39558)

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ntly morphine was found to abolish se-response relationships between strin and gastric acid and pepsin but ie for histamine and cholinergic stim- of gastric secretion (1). It was sug- that this was a consequence of the own morphine interference with ace- re release (2). The explanation ad- was that acetylcholine is an interme- the stimulation of pepsin and gastric gastrin. If this hypothesis is correct, olinium (H3) which depresses acetyl- synthesis should interfere with the of gastrin and pentagastrin (PG) (3). ds. Four dogs, weighing approxi- 20 kg and bearing gastric fistulae and hain pouches, were used in these ex- its. Juice was collected and mea- t 10-min intervals by a wash-out ie from the pouch and by simple e from the fistula.

ed dose-response relationships to ng doses of PG,³ histamine, and pilo- were compared with those achieved after 200 μ g of hemicholinium.⁴ xperiments were conducted on dif- lays. H3 was given as a single iv all the other drugs were given by ous iv infusion.

generalized symptoms of Ach lack pected of H3, in the first few experi- oses were increased by 50- μ g incre- o 200 μ g without apparent toxicity or at any time after the experiments. mpt was made to determine the H3 e.

l stimulation was effected with iv 2- -glucose (2DG), 50 mg/kg in 30 ter 30 min, H3 was given. The sub-

sequent course of secretion was compared with controls without H3.

Acid was titrated using a Radiometer ma- chine and pepsin was estimated by Anson's method (4).

Doses in the dose-response experiments were given for at least three collection pe- riods. Calculations were based on the last two collections of each period. Paired Stu- dent's *t* was used for differences. Dose-re- sponse curves were analyzed for variance between slopes and maximal responses with an Olivetti 602 and its statistical manual. Maximal responses were calculated from re- ciprocal plots.

Results. 2-Deoxy-D-Glucose. The stimula- tory effect of 2DG on acid and pepsin secre- tion from pouch and fistula was diminished significantly by H3 (Fig. 1). Depression in the pouch means either depressed gastrin release from the pylorus or impeded action of gastrin on secretory elements in the pouch or both. This holds also for the fistula with the addition of possible impairment of the direct action of the vagi on fundic secre- tory cells.

Pentagastrin (Figs. 2 and 3). After H3, PG no longer produced progressively in- creasing acid secretion with dose (nonsignif- icant correlation coefficients from either fis- tula or pouch). Fistula pepsin actually showed a decline in secretion with increas- ing PG dose. In Fig. 2, significant lag behind control can be seen at the higher PG doses in the H3 experiments.

Histamine. H3 either significantly aug- mented or left unchanged histamine-stimu- lated acid secretion (Figs. 4 and 5). Pepsin secretion remained unchanged from both parts of the stomach. The histamine V_{max} 's before and with H3 were not significantly different in either pouch or fistula (Table I).

Pilocarpine. When pilocarpine was used as gastric stimulant, pouch acid secretion was augmented by prior treatment with H3 (Fig. 6) and pouch pepsin secretion was un-

orted by NIH Grant No. 5 R01 AM 17125, etion of Pepsin."

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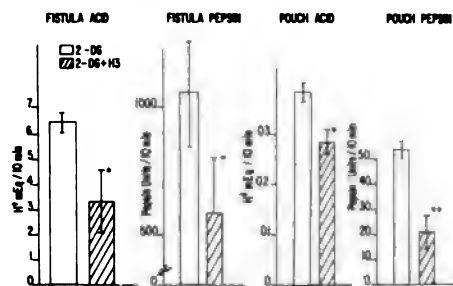


FIG. 1. The effect \pm SE of hemicholinium (H3) on 2-deoxy-D-glucose-stimulated acid and pepsin secretion from gastric fistula and Heidenhain pouch. * $P < 0.05$ for paired difference in four dogs; ** $P < 0.01$ for paired difference in four dogs.

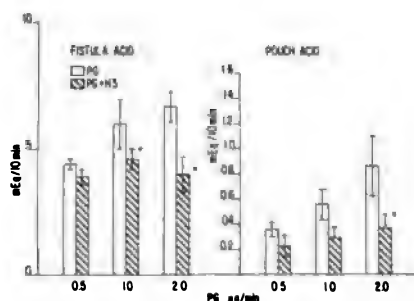


FIG. 2. The effect \pm SE of hemicholinium (H3) on pentagastrin-stimulated acid from gastric fistula and Heidenhain pouch. * $P < 0.05$ for paired difference in four dogs.

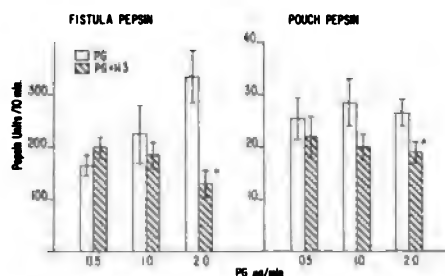


FIG. 3. The effect \pm SE of hemicholinium (H3) on pentagastrin-stimulated pepsin secretion from gastric fistula and Heidenhain pouch. * $P < 0.05$ for paired difference in four dogs.

changed. Fistula secretion after pilocarpine was too contaminated with saliva and duodenal juice to be of value.

Discussion. The depression of 2DG stimulation of gastric secretion and the failure to depress pilocarpine stimulation establish that release rather than action of the cholinergic transmitter is blocked by H3. In view

of the established pharmacological action of H3, this is expected (3).

The finding of H3 depression of PG-stimulated acid and pepsin secretion, but not of histamine-produced secretion or its V_{max} , implies acetylcholine release as an intermediate step in PG but not in histamine action. In fact, H3 often produced augmentation of histamine- and pilocarpine-stimulated acid output. This was the case with morphine also and the fact that morphine is a histamine liberator was advanced to explain it since morphine augmented even resting secretion. This interpretation cannot be used in the present experiments. Alternatively, we propose the existence of a competitive tonic cholinergic suppressor mechanism. In the work of Walsh *et al.* (5), there is suggestion of such. They invoked such a mechanism to explain augmented PG-stimulated acid secretion from Heidenhain pouches after bilateral vagotomy. We invoke it to

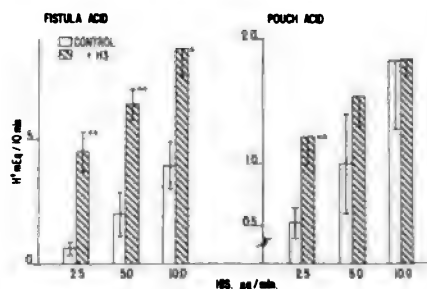


FIG. 4. The effect \pm SE of hemicholinium (H3) on histamine-stimulated acid secretion from gastric fistula and Heidenhain pouch. * $P < 0.05$ for paired difference in four dogs; ** $P < 0.01$ for paired difference in four dogs.

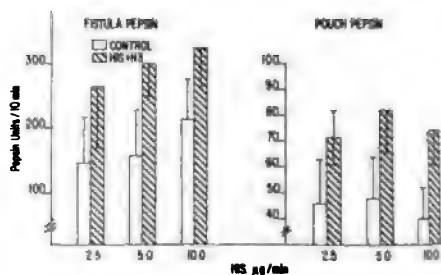


FIG. 5. The effect \pm SE of hemicholinium (H3) on histamine-stimulated pepsin secretion from gastric fistula and Heidenhain pouch. * $P < 0.05$ for paired difference in four dogs; ** $P < 0.01$ for paired difference in four dogs.

TABLE I.

	Fistula		Pouch	
	$1/V_{\max}$	b	$1/V_{\max}$	b
Acid				
Histamine	-0.9	0.414	0.070	0.170
Histamine + H3 ^a	+0.07	0.545	0.430	0.086
Pilocarpine			0.768	0.008
Pilocarpine + H3			0.684	0.014
PG	0.116	1.522	1.080	0.320
PG + H3	—	NS ^b	—	NS
Pepsin				
Histamine	—	NS	—	NS
Histamine + H3	—	NS	—	NS
Pilocarpine	—	NS	0.0063	1.551
Pilocarpine + H3	—	NS	0.0069	0.802
PG	0.0031	115.18	—	NS
PG + H3	0.0088 ^c	-47.35 ^c	—	NS

^a H3 did not produce any other alteration in either slopes or V_{\max} , except that after H3 the PG dose-response relationship was abolished. V_{\max} was, therefore, uncalculable.

^b NS, Nonsignificant correlation coefficients.

^c Significant difference from control; $P < 0.05$.

explain a parallel shift of the histamine dose-response curve to the left of control and an unaltered histamine acid V_{\max} after H3. There is a serious discrepancy, however, in that Walsh *et al.* (5) could not show postvagotomy augmentation of the histamine response.

Since H3, like morphine, produces a suppression of PG-stimulated gastric secretion, the hypothesis advanced in the previous paper (1), that acetylcholine is an obligatory intermediate between PG and acid and pepsin secretion, the latter in innervated mucosa only, is supported.

A characteristic of H3 action is that it is delayed. This is held to be due to the existence of acetylcholine stores which must be used up before the H3-blocking action becomes evident. This is consistent with the finding that only the highest doses of gastrin, which were, of course, the last, showed reatest depression and with the significant negative steps in the PG pepsin dose-response curve after H3.

Summary. Hemicholinium in conscious dogs with gastric fistulae and Heidenhain pouches depressed PG and 2 deoxy-D-glucose stimulation of acid and pepsin secretion. On the other hand, it either augmented or did not significantly change histamine- and pilocarpine-promoted secretion.

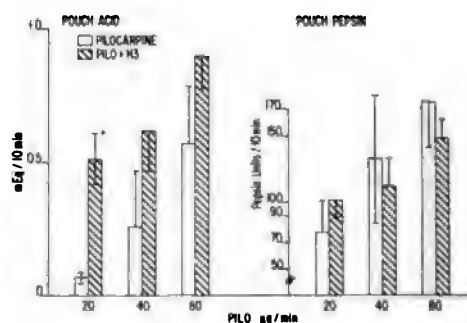


FIG. 6. The effect \pm SE of hemicholinium (H3) on pilocarpine-stimulated acid and pepsin secretion from Heidenhain pouches. * $P < 0.05$ for paired difference in four dogs.

These results support the view that acetylcholine is an obligatory mediator of the action of PG at secretory effector sites.

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The Interrelation of Fluoride and Iron in Anemia¹ (39559)

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A reduced fluoride intake by mice has been reported to increase the severity of the anemia in pregnancy and infancy (1). The anemia has been characterized in 15-day-old mouse pups as a microcytic hypochromic anemia, highly suggestive of an iron or copper deficiency (2). The diet employed in these studies (3) has a high content of whole wheat flour (58%) which contains phytic acid, a chelator of cations capable of interfering with the intestinal absorption of iron (4) and copper (5). The diet has a low fluoride content (0.5 ppm or less), is marginal in iron (29 ppm), and submarginal in copper (2.7 ppm). The requirements for iron and copper for growth of rodents are reported to be 25 and 5 ppm, respectively (6).

Since there are indications that the fluoride effect on the anemia that has been previously reported may be related to iron metabolism, a series of studies with mice on high (50 ppm) and low (deionized water) fluoride aqueous intake, fed the experimental diet supplemented with iron as $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (195 ppm) and the unsupplemented diet, was carried out. In addition to the experimental groups of animals, a control group was fed commercial rat chow (Purina Laboratory) containing 200 ppm of iron, 8 ppm of copper, and 20-60 ppm of fluoride. This group was provided tap water containing 1.0 ppm of fluoride. The effect of the various regimens on the hematocrit value of the newborn and 15-day-old pup was determined. Other regimens with copper supplementation indicated that the copper in the diet did not play an important role in reducing the severity of the anemia. For this reason, the emphasis of this publication is limited to the interrelation of iron and fluoride.

The effect of the maternal fluoride intake

on the total body content of iron was determined in newborn and 15-day-old mouse pups. The dam's milk is the sole source of iron during the first 15 days of life and the measurement of the concentration of iron in the milk of the lactating mouse was considered important in determining the role of this source of iron. The absorption and retention of dietary iron was investigated in animals between the age of 15 and 23 days by providing an oral tracer dose of radioactive iron (^{59}Fe) to each animal in the series.

Experimental procedures. *Experimental Animals.* The mice employed were Swiss-Webster random bred mice placed on the experimental or control regimens as weanlings. All animals were weaned at 20 days of age and mated at 8 weeks of age. Food and water were provided *ad libitum*. At birth, each litter was reduced to six pups to provide a more uniform growth rate for the pups and uniform stress for the nurturing animal. Animals used in the nutritional studies were housed in polypropylene cages with wood shavings provided as litter and nesting materials. Mice involved in the iron absorption studies with radioactive iron (^{59}Fe) were housed in hanging mouse cages to limit coprophagy.

Bleeding and hematocrit determinations. Samples of tail blood were collected in Pre-Cal preheparinized microhematocrit tubes (Clay Adams, Division of Becton, Dickinson, and Company). The tubes were centrifuged for 4½ min in a microhematocrit centrifuge and the hematocrit was determined.

Iron determinations. Five to six grams of diet or the entire bodies of newborn and 15-day-old mice were ashed overnight at 500°. The ash was dissolved in 5 ml of *N* HCl, evaporated to dryness, and reashed at 500° for 4-5 hr. The ash was again dissolved in 1 ml of *N* HCl and made to 50 ml before being analyzed with a Jarrell-Ash atomic absorption spectrometer (7).

The micromethod of Caraway (8) was

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for serum iron determination but was used for the assay of milk since the levels of lipid and protein in milk found to interfere with the assay. A n-free supernate was obtained by using 100 μ l of milk, 50 μ l of 1% ascorbic acid, 0.2 N HCl, 50 μ l of 20% trichloroacetic acid, and 0.05 ml of chloroform. One-hundred-fifty microliters of supernatant was mixed with 0.1% 2,4,6-tripyridyl-S-triazine (TPTZ) solution and the pH was adjusted to between 4 and 5 with 4% ammonium acetate buffer. The TPTZ-iron complex was then extracted with 50 μ l of nitrobenzene, the aqueous layer was discarded, 100 μ l of absolute ethanol was added to the nitrobenzene to reduce surface tension in the microcuvettes used with Beckman DU 25 spectrophotometer could be eased without interfering air bubbles.

Milking. Milk was collected from lactating dams anesthetized with diethyl ether on the ninth or tenth day of lactation after injection of 0.1 ml of oxytocin (2 units). The pups were removed from the dam's cage approximately 6 hr before milking. The mammary glands were massaged, forcing milk toward the nipple so that the drop could be collected in a fine-tipped microcuvette.

Fluoride. The humeri of adult females were removed, cleansed of adhering muscle and tendon, and the bones were split, the soft tissue was removed, dried at 100°C, and then ashed at 500° for 8 hr. The bone ash was analyzed by the method of Singer and Armstrong (9).

Radioiron. A tracer dose of radioactive ^{59}Fe was given by gastric intubation (4 and 0.0188 μ g of iron as FeCl_3 in 0.1 ml) to 15-day-old animals housed with their mothers. Food and water, in accordance with the dietary group assignment, were provided *ad libitum*. On the twenty-third day of life the pups were sacrificed under ether anesthesia after blood had been collected in capillary tubes. After the animal was sacrificed, the gastrointestinal tract was removed. The gastrointestinal tract and the remaining body were placed in separate plastic bags, charred under infrared lamp, and then ashed at 500°. The ash was dissolved in 6 ml of 1 N HCl and was diluted to 5 and 10 ml,

respectively. Sufficient volumes (20–100 μ l) were taken for radioactive counting to provide a reasonable rate of counting. Whole blood (1 ml) samples were ashed at 500°, dissolved by five serial treatments (1 ml) with 1 N HCl, and the final volume was adjusted to 10 ml. Duplicate 100- μ l aliquots of the ashed solution were taken for radioactive counting. Plasma radioactivity was determined with 50- μ l samples pipetted into the scintillation vial. All preparations were counted in scintillation vials containing 15 ml of fluid (0.3% diphenylloxazole in a 70:30 solution of toluene and absolute ethanol) with a Packard Tricarb scintillation counter.

Results and discussion. The fluoride concentrations found in the ashed humeri of dams of the groups (1 year of age) receiving high and low fluoride intake and the diet with and without iron supplementation are given in Table I. Although there is a large difference between the mean fluoride concentration in the bones of adult animals raised on low or high fluoride intakes, it is apparent that supplementation of the diet with iron did not influence the fluoride uptake by bone.

The effect of iron and fluoride intake provided the mothers on the mean hematocrits and weights of 1- and 15-day-old mouse pups is shown in Table II. Animals of all groups had relatively normal hematocrit levels at birth. Iron supplementation of the diet of the dam resulted, however, in an increase in the hematocrit of the newborn as compared to newborn pups of dams provided the unsupplemented diets. Birth weights were similar for all groups. The difference previously reported (1) between the hematocrit for the 15-day-old pups of low and high fluoride mothers raised on the unsup-

TABLE I. FLUORIDE CONCENTRATION IN THE ASHED HUMERI OF FEMALE MICE.

Fluoride intake	Supplement	No. of animals	Percentage F in ash \pm SE
Low	None	10	0.0135 \pm 0.0010
	Fe	17	0.0154 \pm 0.0019
High	None	9	1.23 \pm 0.109
	Fe	15	1.26 \pm 0.045

plemented regimen was observed. Namely, the high fluoride animals (15 days old) had a less severe anemia than animals of the same age from the low fluoride group. There was an apparent beneficial effect on the hematocrit of the young animals resulting from additional iron in the supplemented diet. These later values are similar to those seen for the pups of the control group whose mothers were raised on a commercial laboratory diet (Purina Laboratory Diet).

The total body iron contents of the newborn pups of the control and iron-supplemented experimental groups (Table III) had high and similar levels of body iron stores, whereas the pups from animals maintained on the unsupplemented basal diet had much lower and similar iron contents. The mothers of the low and high fluoride-unsupplemented groups were apparently providing their pups with a smaller iron pool at a time when growth is being highly accelerated and expansion of body fluids is enormous. The total iron present in the 15-day-old pups of dams on a diet unsupplemented with iron and receiving a high fluoride intake was significantly higher ($P < 0.005$) than that of pups of similarly fed dams receiving the low fluoride intake. The high fluoride pups had gained about 198 μg of iron during the 15-

day period as compared to 145 μg for the low fluoride pups. In addition, the serum iron concentration in control (10 animals), high fluoride (18 animals), and low fluoride groups (18 animals) was 381 ± 41 , 276 ± 32 , and 152 ± 8 μg of Fe/ml, respectively. There was a significant difference between the control and high fluoride groups ($P < 0.05$) and between the high and low fluoride groups ($P < 0.0005$). This suggests that (a) there is less dietary iron being provided the low fluoride pups in their early life or (b) there is a more efficient absorption and/or retention of iron from the gastrointestinal tract when the fluoride intake is high.

Mouse pups up until about 15 days of age do not eat the food provided the mother but are nurtured only on milk. It seemed reasonable that, although milk has a low iron content, perhaps the milk from dams of the two experimental fluoride groups might be sufficiently different to account for the difference in iron accumulation during early life as suggested above. The milk obtained from the low and high fluoride dams contained 2.24 ± 0.34 (eight animals) and 3.39 ± 0.22 ppm (nine animals) of iron (mean \pm SE), respectively. It is evident that the fluoride intake of the dam apparently influences the iron content of the milk produced by

TABLE II. EFFECT OF MATERNAL INTAKE ON HEMATOCRITS VALUES AND BODY WEIGHT OF MOUSE PUPS.*

Maternal diet		Newborn			15 Days of age		
Mineral added	Fluoride intake	Number	Hematocrit (%)	Body wt (g)	Number	Hematocrit (%)	Body wt (g)
None	Low	51	42.6 ± 0.78	1.55 ± 0.04^b	36	28.3 ± 0.76	10.7 ± 0.28
None	High	75	41.0 ± 0.65	1.65 ± 0.03	44	33.5 ± 0.96	10.0 ± 0.37
Fe	Low	26	49.3 ± 0.88	1.60 ± 0.03	16	41.1 ± 0.72	10.2 ± 0.31
Fe	High	26	48.9 ± 0.19	1.67 ± 0.05	17	40.1 ± 0.49	9.5 ± 0.36

* Mean \pm SE.

^b Twenty animals of each group.

TABLE III. IRON CONTENT OF MOUSE PUPS.*

Maternal dietary regimen	Newborn		15 Days of age	
	Iron ($\mu\text{g}/\text{pup}$)	Body wt (g)	Iron ($\mu\text{g}/\text{pup}$)	Body wt (g)
Control	81 ± 5.5 (10)	1.60 ± 0.03	338 ± 35.3 (8)	8.3 ± 0.48
Low fluoride	51 ± 2.4 (18)	1.58 ± 0.07	196 ± 11.1 (27)	9.2 ± 0.33
High fluoride	52 ± 2.6 (17)	1.54 ± 0.06	249 ± 12.3 (20)	8.2 ± 0.40
Low fluoride iron-supplemented	83 ± 2.3 (7)	1.46 ± 0.07	335 ± 13.6 (10)	8.0 ± 0.58
High fluoride iron-supplemented	87 ± 2.4 (6)	1.63 ± 0.05	352 ± 41.6 (9)	9.9 ± 0.86

* Mean \pm SE (number).

TABLE IV. RADIOACTIVE ^{59}Fe CONCENTRATIONS IN MICE.^a

Measurement	Maternal dietary regimen		
	Low fluoride	High fluoride	Control
% Dose retained in eviscerated animal	63.5 \pm 1.61 ^b ^c (43)	70.22 \pm 1.33 ^b ^c (34)	79.8 \pm 1.54 ^b (18)
% Dose retained in GI tract	3.52 \pm 0.22 ^b (41)	2.98 \pm 1.25 ^b (33)	1.9 \pm 0.18 ^b (16)
% dose per 0.01 ml of serum	0.058 \pm 0.0045 (38)	0.057 \pm 0.0058 (31)	0.061 \pm .0072 (17)
% Dose per 0.1 ml of blood	7.06 \pm 0.800 (30)	8.47 \pm 0.563 (32)	8.7 \pm 0.70 (18)
Hematocrit at sacrifice	35.0 \pm 0.59 ^b (43)	37.1 \pm 0.74 ^b (33)	42.4 \pm 0.49 ^b (18)

^a Twenty-three days of age. Values are mean \pm SE.

^b Control significantly higher or smaller ($P < 0.005$) than low or high F group.

^c Comparison between low and high F groups, $P < 0.005$. All other comparisons are not significant.

animals fed the unsupplemented experimental diet. Even though the supply of iron in the milk is low, it is the only source available in early life and the difference in iron content between the milk supplies appears to be of major importance. The fluoride content of rodent's milk is not known; however, based on the low levels (0.1–0.2 ppm) reported in milk of other species (10) and the apparent regulation of the fluoride content of milk in normal and fluorosed cattle (11), it can be assumed that the fluoride content of rodent's milk under the conditions of this study is extremely low. If any difference in fluoride intake occurs between the young animals of the two groups during the first 10 days of life, it is insignificant.

The radioactivity (^{59}Fe) remaining in 23-day-old animals 8 days after intubation of the radiotracer dose when the animals were 15 days of age (Table IV) indicates that the control animals absorbed and/or retained a significantly higher amount of the dose in the 8 days following the intubation than did the two experimental unsupplemented groups of animals ($P < 0.005$). Animals on the experimental diet begin to recover from their severe anemic state between 15 and 20 days of age (1). The experimental group of animals receiving 50 ppm of fluoride in the water had significantly ($P < 0.005$) more of the dose in the eviscerated carcass than did the low fluoride animals. The amount of the dose in the gastrointestinal tract was higher for the experimental groups than for the control group ($P < 0.005$), although the total difference in radioiron present was

small. The radioiron is taken up by the mucosa, but it would appear that the iron is not as well utilized by the experimental animals as by the control animals receiving a commercial diet containing 20–60 ppm of fluoride or that the iron stores are being turned over by the experimental animals at a faster rate than is observed for the control animals.

Messer *et al.* in an earlier publication (12) have shown that the fluoride content of the calvaria of the newborn mouse pup of the group given 50 ppm decreased with age up to 10 days of age and was only three times that of the group given the low fluoride diet. By 20 days of age, it was also reported that the fluoride content of the calvaria had increased sharply as a result of the pups being partially weaned and consuming water with a high fluoride content (50 ppm). We have examined the calvaria of 15-day-old pups of the high fluoride group and find that the fluoride content was increased from 0.026 to 0.039% of the ashed bone between 10 and 15 days of age. This circumstance could only occur if the pups had consumed some of the water with a high fluoride content, since the experimental diet had a low fluoride content. The low fluoride group maintained a constancy of fluoride in the bone ash at approximately 0.008%. The young mouse pup although not consuming the food of the dam until approximately 15 days of age, apparently drinks water at an earlier age, and therefore, the 15-day-old animal with dams provided water with 50 ppm of fluoride will consume much more fluoride

than those in the low fluoride group having access to deionized water.

Comparisons of the radioactive results indicate that the control group had a greater absorption and/or retention of the orally administered iron than the experimental groups and that an elevated fluoride intake increased the absorption and/or retention of iron when the iron and fluoride in the prepared experimental diet was minimal. This observation is in agreement with the earlier speculations of Ruliffson *et al.* (13), based on inadequate information, that fluoride intake enhanced the absorption of iron.

The high fluoride animals had a significantly higher concentration of radioactive iron in the blood than the low fluoride animals ($P < 0.025$). This is consistent with the increased production of reticulocytes reported in the high fluoride experimental group over that of the animals receiving the low fluoride regimen or the control animals fed a commercial diet (2).

Summary. It has been demonstrated that iron supplementation can completely prevent the anemia observed in 15-day-old mice raised on a low or high fluoride experimental diet. A higher fluoride intake was again shown to reduce the severity of the anemia seen in low fluoride animals. An increased fluoride intake has been shown to improve iron metabolism when animals were fed an experimental diet marginally adequate in its iron content. The young animals of the high fluoride group were found to have a higher total body iron content at 15 days of age than similar animals of the low fluoride group, although there was no appreciable difference in the iron content of the newborn. The higher fluoride intake of the dam also was shown to increase the iron content of the milk produced by the lactat-

ing dams, and the difference in concentration in the milk from animals in the two groups may account for the observation that there is a higher total body iron burden at 15 days of age in the high fluoride group. In older animals (15–23 days old), the higher fluoride intake was demonstrated to increase the absorption and/or retention of iron by the young mouse provided the experimental diet.

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Cerebroventricular Administration of Leukocytic Endogenous Mediators (LEM) in the Rat¹ (39560)

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systemic injection of rabbit leukocyte-derived factors into rats has been shown to depress plasma iron (1) and zinc levels, increase plasma copper concentration (3), enhance flux of amino acids to liver (4, 5), and increase synthesis of plasma globulins (4). Kampschmidt *et al.* intracisternally injected rabbit LEM into rats and observed a decrease in plasma iron concentration and an increase in blood leukocytes. Intracisternal injections of LEM into rabbits produced a marked fever at lower doses than if given systemically, suggesting a primary site of action in the central nervous system (7). Depending on the procedure of isolation, leukocyte-derived factors have been referred to as endogenous pyrogen, leukocyte pyrogen, or leukocytic endogenous mediator. Because of the multiplicity of activities associated with this crude preparation, the term "leukocytic endogenous mediator" (LEM) was added to these unpurified substances. Rabbit LEM was tested in rat 2B comparison with the majority of the work being done in this area, because Kampschmidt and Church were unable to show a leukocyte pyrogen from rat polymorphonuclear leukocytes (8). LEM obtained from glyco-gen-induced peritoneal exudates in rabbits was injected into the lateral cerebral ventricle of rats in the present study to determine its effects on plasma trace metal concentrations, amino acid flux, and acute-phase

globulin synthesis could be produced by this route.

Materials and methods. LEM (1×10^8 PMN cells/ml) was prepared from rabbit polymorphonuclear (PMN) leukocytes obtained from glycogen-induced peritoneal exudates in the standard fashion (4). A portion of the LEM preparation was inactivated in a boiling water bath for 30 min. Following the boiling period, the crude material was removed by centrifugation (Beckman microfuge for 5 min) and the supernatant represented the heat-treated LEM (Δ LEM).

Male Fisher-Dunning rats with initial body weight of 200 g were used in this experiment. The rats were kept in individual cages with the ambient temperature of $21 \pm 0.5^\circ$. Under sodium pentobarbital anesthesia (40 mg/kg, ip), a guide cannula (Plastic Products Co., Roanoke, Va.) was implanted aseptically into the right lateral cerebral ventricle. The stereotaxic coordinates (9) were 0 mm from the bregma, 1.5-mm lateral to the sagittal suture, and 4.5 mm in depth from the surface of the skull. The rats were given 12 days to recover from the operation before an injection was made according to the procedure of Feldberg and Saxena (10). Another group of rats were injected ip with an equivalent volume of LEM.

Rectal temperature was recorded with a thermistor probe (Yellow Spring Instrument Co., Yellow Springs, Ohio), inserted 6.5 cm into the rectum every 30 min. After the temperature was recorded, animals were placed back in their cages and allowed to move freely.

At various time intervals after the intracerebroventricular (icv) administration of the various substances, the rats were anesthetized with halothane and approximately 1 ml of blood was collected by orbital bleed-

conducting the research described in this report, investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association of Accreditation of Laboratory Care.

ing. A differential and white blood cell count was determined from the heparinized blood sample. The blood was then centrifuged at 3000 g for 10 min in a refrigerated centrifuge. Plasma zinc, iron, and copper concentrations were determined by atomic absorption spectrophotometry (11). α -2-Macroglobulin was analyzed by radial immunodiffusion (12). Some of these rats were pretreated with $[1-^{14}\text{C}]\alpha$ -aminoisobutyric acid ($[^{14}\text{C}]\text{AIB}$) so that amino acid flux to the liver could be determined according to the method of Wannemacher *et al.* (13). Statistical analyses were done using Student's *t* test for paired or unpaired variates.

Results. Figure 1 illustrates the hyperthermic response to icv LEM (20 $\mu\text{l}/\text{rat}$) in 20 different rats. Temperature began to rise (2–3°) steeply within 60 or 90 min of each injection. The hyperthermic response was relatively long-lasting but did not exceed 24 hr. Heat-treated icv LEM (20 $\mu\text{l}/\text{rat}$) did not alter normal rectal temperatures. Heated endotoxin (Δ endotoxin; boiling in water bath for 30 min) and endotoxin (10

ng/20 $\mu\text{l}/\text{rat}$) both produced an icv hyperthermic response (1–2°) which began to rise 90 or 120 min after the injection. The endotoxin hyperthermic response appeared to have the same duration as that caused by LEM. After injection of 10 or 50 μl of icv LEM, the hyperthermic response was similar to that after 20 μl of icv LEM (not shown).

As shown in Fig. 2, a single injection of icv LEM (20 μl) produced a significant ($P < 0.001$) depression in plasma iron concentrations which was evident within 1 hr, reached an apparent nadir at 7 hr, and gradually started approaching, but did not achieve, control values by 24 hr. Plasma zinc concentrations also showed a significant ($P < 0.01$) decrease 3 hr postadministration icv with maximum depression occurring in 5 to 7 hr ($P < 0.001$). At the 24-hr period, plasma zinc was still significantly ($P < 0.01$) lower but had started to approach control

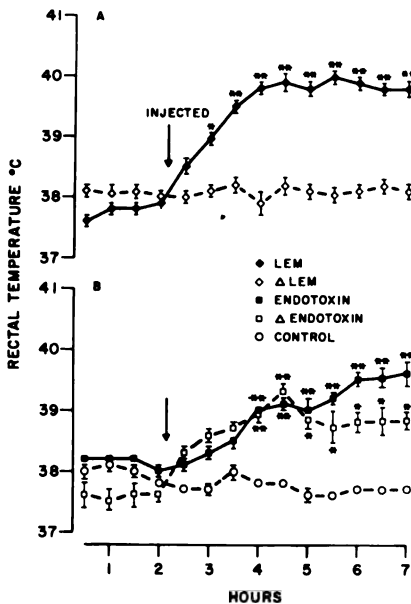


FIG. 1. Rectal temperatures induced by injecting 20 μl of LEM (\blacklozenge , $n = 20$), Δ LEM (\diamond , $n = 10$) (panel A), endotoxin (\blacksquare , 10 ng/20 μl , $n = 10$), Δ endotoxin (\square , 10 ng/20 μl , $n = 5$), or saline (\circ , $n = 5$) (panel B) icv into unanesthetized, unrestrained rats. Values are mean \pm SE. * $P < 0.01$, ** $P < 0.001$ vs Δ LEM or saline.

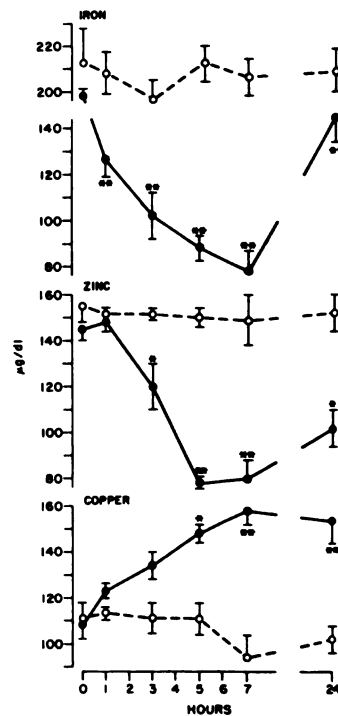


FIG. 2. Effect of icv LEM (20 μl) on plasma Fe, Zn, and Cu concentrations. Open circles represent control values of combined normal and Δ LEM rats. Closed circles represent LEM administration. Values are mean \pm SE of five to eight rats. * $P < 0.01$, ** $P < 0.01$ vs controls.

(Fig. 2). Plasma copper concentrations were observed to be significantly elevated at the 5- ($P < 0.01$), 7- ($P < 0.001$), and 24-hr ($P < 0.001$) periods (Fig. 2).

LEM and saline given icv did not affect plasma iron, zinc, or copper concentrations from those of normal rats. Temperatures were also obtained from these rats after icv injection and the results are similar to those presented in Fig. 1.

In order to determine the time sequence of plasma α_2 -MFP response, rats were given icv with 10, 20, or 50 μ l of LEM. Animals were bled from the orbital venous plexus at 0-, 5-, 24-, and 48-hr intervals after the injection; the plasma was assayed by radial immunodiffusion. The results are presented in Table I. Five hours after injection of LEM, no significant change in α_2 -MFP was observed. LEM in a icv dose of 10 μ l failed to significantly elevate α_2 -MFP at the 24- and 48-hr periods. Significant increases in α_2 -MFP were observed at icv doses of 20 and 50 μ l at the 24- and 48-hr periods. Orbital bleeding may have been responsible for the slight increase in α_2 -MFP in animals given saline or heated LEM.

Four weeks after the initial icv administration

of LEM, a 20- μ l dose of saline was given and the temperature was monitored in order to determine if hyperthermia occurred.

Four days after the saline injection, the rat was injected sc with 1 μ Ci (0.1–0.2 μ Ci/100 g body weight) of the 14 C-labeled amino acid 24 hr prior to use (17). At 0800 hr on the next day, rats

were injected icv with 20 μ l of LEM, heated LEM, or saline. At various time intervals (1, 3, 5, and 7 hr) after the icv injections, a 1-g sample of liver was removed from four individual rats at each time period for determination of hepatic concentration of [14 C]AIB. As shown in Fig. 3, LEM significantly increased the hepatic concentrations of [14 C]AIB. The increase was evident within 1 hr and remained elevated by the 7-hr period when compared to heated LEM values. Intraperitoneal injections of an equivalent volume of LEM (10, 20, and 50 μ l) had no effect on the measured parameters (not shown).

Discussion. In the present study LEM (10–50 μ l) was found to cause hyperthermia when injected into the lateral cerebral ventricle of the rat; these doses were inactive when administered ip or iv. In contrast, Kampschmidt and Upchurch observed rabbit LEM to produce hypothermia in rats following an ip injection. The fact that heat treatment abolished the hyperthermia produced by LEM but not that of endotoxin is good evidence that the LEM preparations were not contaminated by endotoxin (4). Additional evidence that endotoxin was not present in the LEM preparation was that LEM produced a more rapid onset of hyperthermia than did endotoxin.

Alterations in serum zinc, iron, and copper concentrations after ip injection of 1.0 ml of LEM have been well documented. This study demonstrates for the first time that icv injections of LEM cause a decrease in serum zinc and iron and increases in cop-

TABLE I. EFFECTS OF VARIOUS DOSES (10–50 μ l) OF ICV LEM ON PLASMA α_2 -MFP CONCENTRATIONS.*

Treatment	Hours			
	0	5	24	48
Saline	0	0	3.0 \pm 1.0	4.8 \pm 0.5
LEM	0.2 \pm 0.1	0.4 \pm 0.3	4.0 \pm 2.0	6.0 \pm 4.0
Δ LEM	0.9 \pm 0.4	8.0 \pm 8.0	5.0 \pm 3.0	4.0 \pm 2.0
Saline	0	0	4.0 \pm 4.0	8.0 \pm 7.0
LEM	0.4 \pm 0.4	0.6 \pm 0.5	24.2 \pm 4.0**	24.2 \pm 3.0*
Δ LEM	3.3 \pm 2.0	2.5 \pm 2.0	6.6 \pm 2.0	8.0 \pm 8.0
Saline	0	0	2.0 \pm 1.0	3.5 \pm 3.0
LEM	0.1 \pm 0.1	7.0 \pm 5.0	34.8 \pm 10.0*	33.4 \pm 12.0
Δ LEM	0.9 \pm 0.6	0.1 \pm 0.02	6.1 \pm 2.0	4.4 \pm 1.0

* are mean \pm SE of four to five rats in units per milliliter.

0.05 vs heated LEM; ** $P < 0.01$ vs heated LEM.

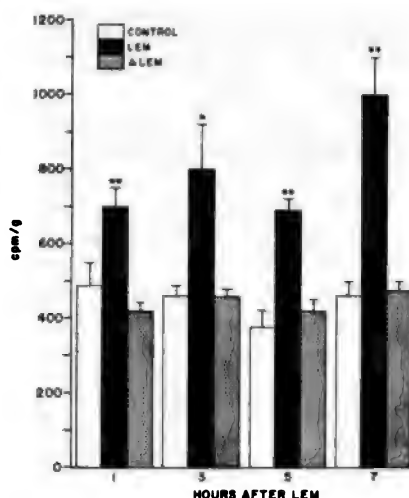


Fig. 3. Effects of icv LEM on hepatic concentration of [^{14}C]AIB. Rats were injected icv with either 20 μl of LEM, ΔLEM , or saline. Values are mean \pm SE of four rats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs controls.

per concentrations. Kampschmidt *et al.* (6) also observed changes in serum iron after intracisternal injections of 10 μl of LEM. These data are additional evidence of central nervous system (CNS) involvement in the action of LEM. Systemic (ip) administration of LEM has been shown to elevate the concentration of acute-phase globulins and stimulate an increased rate of transport of nonmetabolizable amino acids from the plasma to the liver (4) in rats. The present study demonstrates that an icv injection of LEM also leads to a heretofore undescribed CNS role in the elevation of an acute-phase protein (α_2 -MFP) concentration and a marked hepatic intracellular accumulation of a nonmetabolizable amino acid. Thus, LEM can act via a CNS mechanism in stimulating the hepatic transport of amino acids and the synthesis of plasma α_2 -MFP. In contrast, *in vitro* data suggest that LEM may act directly on liver cells to stimulate increased transport of amino acids (5).

The current studies have demonstrated that LEM in doses which are inactive ip can mediate certain effects on or through the CNS, thereby supporting the hypothesis that LEM may have a primary site of action in the CNS.

Summary. LEM obtained from glycogen-induced peritoneal exudates in rabbits were injected into the lateral cerebral ventricle of rats to determine if LEM had a primary site of action in the central nervous system. LEM injected icv in the dose of 10, 20, or 50 μl was observed to produce a rapid hyperthermia. Injection of heated LEM failed to cause a hyperthermic response. Endotoxin and heated endotoxin (20 ng/20 μl /rat) administered icv each produced a delayed hyperthermia as compared to LEM. The various doses of LEM were also observed to significantly decrease plasma iron and zinc, increase plasma copper, increase the synthesis of plasma α_2 acute-phase protein (α_2 -MFP), and cause a flux of a nonmetabolizable amino acid ([^{14}C]AIB) to the liver. These observations suggest that LEM in doses which are inactive systemically can mediate certain effects on or through the central nervous system following icv administration.

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Canine Cyclic Hematopoiesis: Platelet Size and Thrombopoietin Level in Relation to Platelet Count¹ (39561)

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Data from several laboratories have indicated that young platelets are larger and heavier than other platelets (1-7). Several workers (1-6) have shown that these platelets become smaller and lighter with age. However, in one study (7), it was claimed that large platelets produced following acute blood loss did not decrease in volume as they aged. These workers proposed that large platelets obtained from acutely bled dogs are "stress" platelets and may represent an aberrant population. Biochemical data support the finding of a decrease in platelet size with age (8). However, kinetic data neither absolutely prove that large-heavy platelets are the only young platelets nor rule out the possibility that large-heavy platelets might be "stress" platelets.

Cyclic hematopoiesis (CH) in dogs has been extensively studied in several laboratories (9-11). This syndrome in dogs serves as a model for studying cyclic thrombopoiesis disorders in humans (12, 13). Previously it has been established that in CH dogs cycling of platelets occurs at 11- to 14-day intervals and that platelet values cycle from near normal to above normal values (14). Since platelet counts cycle in these dogs and platelet size is thought to be related to the age of an individual platelet, it seemed possible that average platelet size might fluctuate with changing age composition of the platelet population as the proportion of young platelets changed.

Plasma levels of a thrombopoiesis-stimulating factor (TSF or thrombopoietin), a humoral agent that controls blood platelet production, were shown to fluctuate in a patient with cycling thrombopoiesis (13). This worker presented evidence in favor of the

hypothesis that TSF deficiency may cause platelet counts to cycle in humans. In an attempt to clarify the causes of cycling platelet counts in CH dogs, TSF measurements were made on plasma fractions.

Materials and methods. Littermate CH dogs (Nos. 489, 490, 491) and a normal control littermate dog (No. 492) were used in the sizing studies. Throughout the study, less than 3 ml of blood were taken per day from these dogs (weighing about 6 kg at the beginning of the experiment). In another study, a CH dog (No. 347, weighing 10.5 kg) and a normal control dog (No. 415, weighing 10.9 kg) were used to determine the TSF levels associated with thrombopoiesis, and in these dogs 30 ml of blood were collected each day.

Platelet counts (determined by direct counting under phase-contrast microscopy) were made on blood taken from the jugular vein into vacutainers containing EDTA. For size measurements, platelets were taken from other samples of blood diluted into heparin. Approximately 0.5 ml of blood from each dog was transferred into a plastic tube (12 × 75 mm) containing 1 ml of a saline-heparin solution (100 units heparin), and the platelet-rich plasma (PRP) was separated by centrifugation at 450g for 4.5 min at 22°. The PRP was then removed and diluted with Isoton (Coulter) to a concentration of 12,000 to 18,000 platelets/100 μ l for size analysis.

Average platelet sizes on a logarithmic scale were determined by use of an ElectroZone/Celloscope (Particle Data, Inc.) equipped with a 128-channel analysis accessory and direct readouts to an X-Y oscilloscope, and an X-Y plotter. The instrument settings were: log 10 (logarithmic span of about 10 doublings of particle volume or a 10:1 in diameter); current, 2^{1/2}; and gain, 17. The multichannel analyzer was set for acquisition to a count of 4000 in

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peak channel. Calibration was maintained at all times via frequent checking of particles of known size. Triplicate samples were sized, size distributions were plotted by use of the X-Y plotter and size (microns diameter) was calculated as previously described (15).

In the TSF study, CH dog No. 347 and a normal control dog (No. 415) were bled from the jugular vein at daily intervals for 5 days. In all cases, a standard volume of 30 ml of blood was collected from each dog into a conical centrifuge tube containing 1 ml of citrate-phosphate-dextrose (CPD) solution (citric acid, 4.57 g; sodium citrate, 5.0 g; NaH_2PO_4 , 3.1 g; dextrose, 35.66 g/100 ml of water). The plasma from each dog was then subjected to fractionation using E-phosphate cellulose column chromatography (16). This technique has been found to be useful for concentrating the platelets. Prior to chromatography, the plasma from each bleeding of the dogs was dialyzed for 3 hr against three changes of distilled water, adjusted to pH 5.5, and then added to a column containing DEAE-phosphate cellulose, pH 5.5. The TSF-rich fraction was eluted from the column with 1.0 M NaCl in 0.1 M Na_2HPO_4 buffer, pH 8.2 (Step I dog plasma TSF). After elution, the fraction was dialyzed against distilled water and lyophilized to dryness.

The lyophilized Step I dog plasma was resuspended into saline and assayed for TSF activity by use of thrombocythemic mice. Five days before injection of the Step I plasma preparations (extracted from both normal dog plasmas), the assay mice were given a single ip injection of rabbit anti-mouse platelet serum (RAMPS). PS was produced in rabbits as described previously (18). This antiserum produced a characteristic thrombocytopenia at 4 hr which was followed by rebound thrombocytosis. While thrombocythemic, the dogs were injected sc with 20 mg of protein extract of Step I dog plasma preparations, two times on Day 5 after the RAMPS injection, and two times again on Day 6. Thirty Curies of $\text{Na}_2^{35}\text{SO}_4$ in 0.5 ml of saline was injected iv on Day 7 and the percentage of ^{35}S -incorporation of injected dose circulating platelets was measured 24 hr

later (Day 8) in blood samples obtained by cardiac puncture.

The platelet ^{35}S incorporation data and platelet size measurements were subjected to Student's *t* test.

Results. Figure 1 shows the results of de-

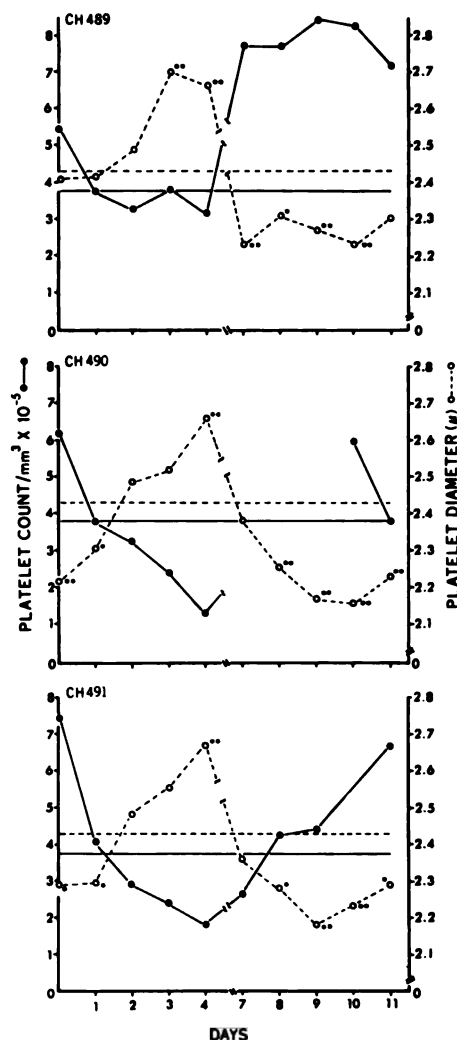


FIG. 1. Platelet counts and platelet sizes of four littermate dogs measured during a 2-week period: CH dogs, No. 489, No. 490, and No. 491, and a normal dog, No. 492. Horizontal dashed lines represent average platelet size and the solid lines show the average platelet count for the normal dog. Platelet sizes for the normal dog varied from 2.34- to 2.49- μm diameter; platelet counts were from 3.12 to $4.0 \times 10^3/\text{mm}^3$ for the 2-week period. Platelet sizes were significantly different from normal control: * $P < 0.005$; ** $P < 0.0005$.

termining platelet sizes and platelet counts on three CH dogs and one normal dog during a 2-week period. The data show highly significant changes with time in platelet sizes for all three CH dogs. Moreover, there was an inverse relation between platelet size and platelet counts, i.e., when platelet counts were normal or below normal, the average platelet sizes were significantly larger than platelets from a normal dog. Conversely, when platelet counts in CH dogs were elevated above normal values, the average platelet sizes were decreased to significantly below the normal control dog values.

Figure 2 shows the results of platelet counts of a CH dog and a normal dog during a 5-day period of time. Also shown are the values for percentage of ^{35}S incorporation into platelets of assay mice after injection of

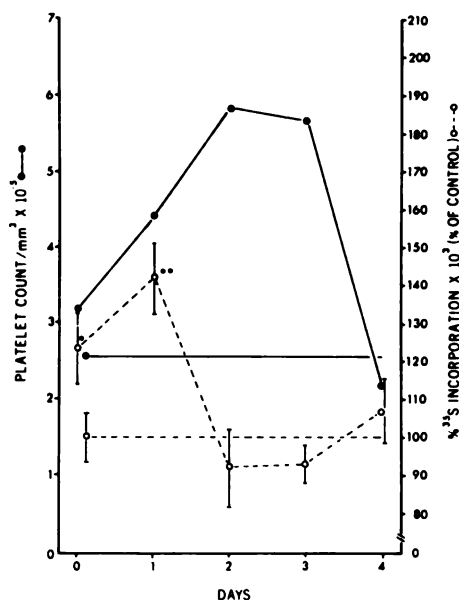


FIG. 2. Platelet counts of a CH and a normal dog, and TSF assay of plasma samples collected daily for 1 week. Percentage of ^{35}S -incorporation values was obtained from five to six mice after injection of 20 mg of plasma extracts at each point; vertical lines represent the standard error. Horizontal solid line represents the platelet count of the normal control dog, and horizontal dashed line represents the percentage of ^{35}S -incorporation of 15 mice injected with a pool of plasma extracts collected daily from a normal dog. Platelet counts of normal dog varied from 2.40 to $2.68 \times 10^5/\text{mm}^3$. ^{35}S values were significantly elevated over control values: * $P < 0.05$; ** $P < 0.01$.

fractions of dog plasma from the two dogs. The platelet count of the CH dog on Day 0 was only slightly above that of the normal dog; platelets of the CH dog then increased at a rapid rate and at 2 days reached two times the level found in the normal dog. The platelet count leveled off by Day 3 and decreased drastically by Day 4. Plasma TSF levels were elevated at the beginning of active thrombopoiesis on Days 0 and 1 ($P < 0.05$ and $P < 0.01$, respectively). However, when the platelet counts were elevated to approximately two times the normal control count, the TSF levels dropped below (not statistically significant at the 0.05 level) the TSF levels of the normal control dog and remained low for another day. When the platelet count dropped to below the normal level (on Day 4), the TSF titer once again increased to slightly above normal levels.

Discussion. Cyclic hematopoiesis in dogs has been extensively studied by several investigators (10, 11, 14, 19) and this syndrome in dogs serves as a useful model to compare with a similar disorder in humans, cycling thrombopoiesis (12, 13). Although the cause of the disorder in dogs remains to be determined, it seems clear that platelets cycle from about 125,000 to 900,000/ mm^3 and the cycles occur at 11- to 14-day intervals (9, 19-21). In preliminary experiments, several littermate control dogs of this strain had platelet counts of 200,000 to 500,000/ mm^3 , with a small amount of variation from day to day. Also, no differences in platelet counts were observed in dogs heterozygous for the CH gene when compared to other dogs that were proven to be free of the gene. Platelet sizes and thrombopoietin levels have apparently not been reported previously in CH dogs.

The results of several erythropoietin studies in CH dogs have been presented recently (22-24). Although reticulocytes appear to cycle at regular intervals, erythropoietin could not be detected in dog plasma without stimulation by hypoxia (23) or bleeding (24). Lange and Jones (22) hypothesized that oscillations of erythropoiesis and thrombopoiesis might result from the production of a "poietin-controlling-factor" that stimulates the production of specific factors (i.e., erythropoietin, etc.) leading to in-

in reticulocytes, platelets, and monocytes. These authors thought that such a sequence of events would explain the appearance of platelet counts at a time when the granulocyte counts are reached. They postulated that feedback mechanisms must be present.

The present work describes an animal in which average platelet size changes in concert with cycling of platelet production. It should also be mentioned that *et al.* (25), employing a sucrose density gradient, noted a larger percentage of platelets following recovery from thrombocytopenia in a patient with thrombotic deficiency (12). It, therefore, is possible that CH dogs will be useful in studying the causes of cycling thrombotic disorders in man.

Winter and Ingram (7) have proposed that large-heavy platelets obtained from bled dogs are "stress" platelets and these platelets do not decrease in size with age. Other workers (1-6) have presented data that are consistent with a decrease in platelet size with age. In the present work, large platelets were found in unbled dogs when the platelet counts were only slightly below normal. It was noted that blood loss was not a factor responsible for changes in number and size of platelets because the blood sample was 3 ml or less per day, which is less than 1% of the total blood volume of these dogs. Four to six days after maximum size, average platelet sizes were significantly smaller than platelet sizes from a normal dog. Dogs are reported to have platelet life spans of about 8 days (26). However, the data of the present report do not conclusively prove that platelets in these dogs decrease in size with age, since platelet life spans were not measured.

It should be mentioned that in the present work platelet size increased while the platelet counts were decreasing. These results indicate that when platelet counts are low, megakaryocytes are releasing large platelets and when platelet counts are high, platelets are either being produced or removed from the circulation as a result of increased thrombopoiesis, possibly by a result of negative feedback on TSF levels. Further

work, therefore, is needed in these dogs to clarify these results. It also seems possible that large platelets produced by stress (such as bleeding) might behave differently from platelets that are normally produced.

Regardless of whether large platelets are normally produced or "stress" produced, they can be conveniently employed to predict megakaryocyte numbers and, hence, platelet production (27). Moreover, it is well known that bleeding releases erythropoietin, and Jackson *et al.* (28) have proposed that erythropoietin might be an inducer of thrombopoiesis. In fact, some experiments (29) have shown that blood loss and/or iron deficiency has an effect on thrombopoiesis. It therefore seems possible that erythropoietin might have, in previous studies, produced "stress" platelets. In the present work, which demonstrated remarkable changes in platelet size without bleeding, erythropoietin was apparently not the cause of increased platelet sizes, but large platelets were probably the result of thrombopoietin action in dogs with cycling thrombopoiesis.

Previously, Weiner and Karpatkin (4), employing a thrombopoietic stimulus (injection of plasma from thrombocytopenic guinea pigs into recipient animals), showed a 2.7-fold increase in numbers of large platelets compared with a 1.5-fold increase in platelet counts after a 4- to 5-day lag period. These experiments were confirmed in rabbits (6). Whereas these workers (4, 6) used exogenous sources of TSF and found changes in platelet sizes, we have shown here both decreased platelet sizes in dogs and increased levels of endogenous TSF at a time when the platelet count was being regenerated (active thrombopoiesis). Moreover, TSF was not detected in the circulation when platelet counts of the dog rose to more than two times the normal count. The data of the present work agree with previous reports indicating that average platelet size increases in animals with TSF-stimulated thrombopoiesis.

Severe cyclic thrombocytopenia has been reported (13) in a young woman. This rare phenomenon is of considerable theoretical interest in relation to platelet kinetics. Plasma TSF levels were measured and the

results suggested that TSF deficiency was the underlying cause of the disease. The data of the present work agree with the finding in the human case of increased TSF at the beginning of active thrombopoiesis. Also, the plasma TSF levels of a CH dog were below normal (but not significantly) when the platelet count was about two times the normal count. Whether TSF deficiency causes platelet cycling in dogs, however, remains to be proven.

Summary. Platelet size, platelet count, and thrombopoietin levels in plasma fractions were determined in cyclic hematopoietic (CH) dogs. Results show that platelet sizes varied inversely with platelet counts of CH dogs; significantly elevated levels of thrombopoietin were found in fractions of plasma from CH dogs at the beginning of active thrombopoiesis. These dogs serve as a useful model for studying cycling thrombopoiesis in man.

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Effect of Epinephrine on the *in Vivo* Concentration of Erythrocyte Glycolytic Intermediates (39562)

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Studies reported herein were initiated because we observed an increase in the concentration of erythrocyte adenosine triphosphate (ATP) in a rhesus monkey which escaped from its cage and was recaptured after a 10- to 15-min chase. This observation led to experiments, which will be reported in this paper, dealing with the effect of epinephrine injections on rhesus erythrocyte glycolytic intermediate concentrations. A pharmacological agent which increases red cell ATP might accomplish this by stimulating erythrocyte glycolysis. Stimulation of glycolysis can potentially increase erythrocyte ATP and 2,3-diphosphoglycerate (DPG) concentrations which in turn may enhance erythrocyte function. ATP is generated by the glycolytic process and is important in maintaining normal shape (1, 2), deformability (3) of the red cell. The cell also uses ATP to maintain physiological intracellular concentrations of Na^+ , K^+ , and Ca^{2+} . DPG, another important molecule generated by glycolysis, increases release of oxygen from hemoglobin to tissues by decreasing the oxygen affinity of hemoglobin (5, 6).

Materials and methods. In initial experiments, male rhesus monkeys were anesthetized by intramuscular injection of phencyclidine hydrochloride (Sernylan, Parke-Davis Co.). In later experiments, the monkeys were anesthetized by a combination of ketamine hydrochloride (Ketalar, Parke-Davis Co.) intramuscularly at a dose of about 20 mg/kg body weight and pentobarbital given slowly by the intravenous route at a dose of 5-10 mg/kg body weight. Pentobarbital alone was found to be just as effective as the combination of anesthetics and was used alone in most experiments. The type of anesthetic used is designated by letters in the tables.

One milliliter of epinephrine solution (1

mg/ml) was given intraperitoneally to anesthetized monkeys, followed 15 min later by another 1.5 ml. All of the monkeys studied weighed about 5.0 kg, thus the 3.5 mg of epinephrine represented about 0.7 mg/kg. Controls were injected with an equivalent volume of saline. About 12 ml of blood were drawn from anesthetized monkeys 30 min after the first epinephrine or saline injection. The blood was drawn into heparinized syringes via puncture of the femoral vein and a portion precipitated immediately for the determination of ATP, DPG, and glycolytic intermediates. In some of the early experiments, glycolytic intermediates were not assayed.

The blood precipitation procedures have been described previously and employ trichloroacetic acid for the precipitation of ATP and DPG, and perchloric acid for the precipitation of the remaining glycolytic intermediates. The assay of ATP follows the method of Brewer and Powell (7) and the DPG assay is that of Keitt (8). The method of preparation and assay of extracts for the remaining glycolytic intermediates is a modification of Oelshlegel *et al.* (9) of that described by Minakami *et al.* (10). Leukocytes and platelets are not removed before the blood is precipitated because the contribution of these cells to the concentrations of glycolytic intermediates in blood has been found to be negligible (10).

In some of the early epinephrine injection experiments, the blood used to determine control levels of ATP, DPG, and glycolytic intermediates was drawn from the same monkeys 9 days before or after the blood used to determine epinephrine-treated levels of these variables. This scheme was initially employed because we suspected that removal of the blood *per se* for determination of control values might influence the

level of variables of interest in blood drawn minutes later for postepinephrine values. This precaution was found to be unnecessary, so in subsequent experiments the control blood was drawn from anesthetized monkeys 15 min before the first epinephrine injection, and then the usual protocol was followed.

Epinephrine-induced hyperventilation might stimulate glycolysis via alkalosis and thus would complicate the interpretation of the epinephrine effect. Therefore, in two experiments, anesthetized monkeys were intubated endotracheally in order to maintain a constant blood pH during the subsequent epinephrine treatment. In one of these experiments, the animal breathed air supplemented with 3% CO₂ for this purpose, and in the other, a small animal respirator was used to control respiration during the course of the experiment.

Results. As mentioned above, we observed that red blood cells from a monkey which escaped and was chased vigorously for 10–15 min had a much higher ATP concentration than was found in the same monkey's red cells when blood was taken under normal resting conditions. In three pilot experiments, we found deliberate vigorous (but not slow) chasing of monkeys also increased their red cell ATP concentration (data not shown).

We did not extend these qualitative observations by obtaining enough data for statistical testing because the chasing experiments were difficult for both animals and investigators. Instead, we turned to experiments designed to determine if epinephrine injected ip into monkeys would produce a similar change in red cell ATP concentration. Table I presents the results of 11 experiments using six 5-kg male monkeys injected with 3.5 mg of epinephrine as described in the Methods section. From Table I it is apparent that epinephrine injections produced a consistent and statistically significant increase in red cell ATP concentration. The average values of control and postepinephrine blood samples were 4.77 and 5.70 μ mole of ATP per g of hemoglobin, respectively. The average increase in ATP concentration was 19.5% and the difference between control and epinephrine

ATP mean concentrations is significant at the 1% probability level ($P = 0.0144$). The DPG response varied in these experiments and no significant change could be discerned. Prevention of hyperventilation by use of a respirator, or respiration with 3% CO₂ to prevent alkalosis, did not block the increase in ATP induced by epinephrine (Table I).

The effect of epinephrine injection on the levels of glycolytic intermediates in rhesus monkey erythrocytes has been studied in several experiments in order to identify the enzymatic step or steps responding to epinephrine. These experiments are presented in Table II. Epinephrine injection consistently increased the concentration of early red cell glycolytic intermediates, glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), dihydroxyacetonephosphate (DHAP), as well as increasing the ATP concentration. The mean fructose 1,6-diphosphate (FDP) for the six experiments is also increased. Experiment 4 is a time-course experiment for the effect of epinephrine on the glycolytic intermediates. The two injections of epinephrine were followed by a sequence of blood drawings at the usual 30 min after the first injection; in addition, blood was also drawn at 60 and 120 min after the first injection. It is apparent that G6P and ATP levels continued to increase throughout the experiment. In experiment 5, the monkey was intubated endotracheally and ventilated with air supplemented with 3% CO₂ in order to maintain constant blood gas and blood pH during the epinephrine treatment. From the data in Table II, it is evident that G6P, F6P, FDP, DHAP, and ATP are increased subsequent to the epinephrine injections under conditions where the blood CO₂ level is being controlled. In experiment 6, hyperventilation (and alkalosis) was prevented by controlling ventilation at 1.4 liters (40 breaths) per min throughout the experiment by a small animal respirator. Again G6P, F6P, FDP, DHAP, and ATP were elevated after the epinephrine injections. The whole blood pH values presented in the treatment column of Table II (experiments 5 and 6) indicate that respiratory alkalosis is not required for the elevation of G6P, F6P, FDP, DHAP, or

TABLE 1. THE EFFECT OF EPINEPHRINE INJECTIONS ON THE CONCENTRATION OF ATP AND DPG IN MALE RHESUS MONKEY RED CELLS.

Monkey No.	ATP (μ mole per g of hemoglobin)		DPG (μ mole per g of hemoglobin)		Hemoglobin (g%)	
	Control	Epinephrine	Control	Epinephrine	Control	Epinephrine
266 ^a	3.75	5.33	—	—	12.64	12.50
269 ^a	4.33	5.17	16.21	18.08	12.99	14.11
283 ^a	5.93	6.14	16.14	16.81	11.37	12.36
284 ^a	4.75	5.31	19.83	19.85	11.94	11.73
286 ^a	4.92	5.31	19.66	19.51	10.81	14.11
286 ^a	4.67	8.45	20.16	19.20	11.02	13.90
286 ^b	4.68	5.68	19.28	24.97	11.72	10.25
287 ^a	4.70	4.87	19.51	17.42	12.64	12.15
287 ^b	4.22	4.48	14.00	15.94	14.01	14.55
284 ^{b,c}	5.98	6.29	24.04	24.11	8.64	11.02
286 ^{b,d}	4.54	5.71	16.60	20.56	11.23	12.78
Mean	4.77	5.70	18.54	19.64		
Mean difference \pm SE ^e	0.93 \pm 0.31		1.10 \pm 0.74			
Unpaired Student's <i>t</i> statistic	2.956		1.49			
Significance of difference	<i>P</i> = 0.0144		<i>P</i> = 0.169			

^a Control and epinephrine treatment on different days.

^b Control blood drawn 15 min before first epinephrine injection.

^c Respirator experiment, control blood pH was 7.470; postepinephrine pH was 7.338.

^d Three-percent CO₂ experiment, control blood pH was 7.363; postepinephrine pH 7.358.

^e SE = Standard error.

Note. Since some of the monkeys were used in more than one experiment, the mean control values was compared to the mean treatment value for each monkey. The average increase in ATP concentration was 17% (*P* = 0.026).

TP. The phosphorylated intermediates in the lower part of the glycolytic pathway (3-phosphoglycerate and phosphoenolpyruvate) do not change in any consistent way after epinephrine injection. In all of these experiments, except experiment 3, the adenosine diphosphate (ADP) has decreased subsequent to epinephrine injection. The total adenine nucleotide pool has increased except in experiment 6 where the pool decreased by 2.4% due to the relatively large decrease in ADP. The mean increase in the adenine nucleotide pool is 0.9%. This increase derives from the increased ATP.

Blood pyruvate and lactate are a result of metabolism in blood cells and various other tissues in the body, since these metabolites cross cell membranes. Modest increases in blood pyruvate and lactate concentration are generally found subsequent to the epinephrine except in experiment 5 of Table II (the 3% CO₂ experiment) where both pyruvate and lactate decreased in concentration. The lactate/pyruvate ratios have not been tabulated, but this ratio consistently de-

creased following the epinephrine treatment. In experiment 3, the lactate/pyruvate ratio after treatment was only 12% of its control value.

In two experiments the epinephrine-induced increases in the concentrations of red cell G6P, F6P, and ATP were not delayed or reduced by the administration of 300 mg of aspirin plus 7.5 mg of indomethacin to a 6.5-kg monkey at 4 hr and again at 15 min before the initial blood was drawn for determination of control levels of glycolytic intermediates (data not shown). In two additional experiments, using one-tenth as much epinephrine (0.35 ml of 1 mg/ml of epinephrine injected into each of two monkeys weighing 4.8 and 6.5 kg), no changes occurred in the glycolytic intermediates or ATP (data not shown). Subcutaneous injection of monkeys with prostaglandin E₂ (in 95% ethanol) at doses of 3 mg/5.25-kg monkey and 5 mg/6.5-kg monkey did not cause a consistent change in G6P, F6P, or ATP (data not shown).

Discussion. In 11 different *in vivo* experiments using six 5-kg rhesus monkeys in-

TABLE II. THE EFFECT OF EPINEPHRINE ON MONKEY ERYTHROCYTE GLYCOLYTIC INTERMEDIATES.

Expt. No.	Monkey No.	Treatment	Concentration in $\mu\text{mol/g}$ of hemoglobin ^{a,b}											
			G6P	F6P	FDP	DHAP	DPG	3PG	PEP	PYR	LACT	AMP	ADP	ATP
1 ^c	286 (4.8 kg)	Control/ Epinephrine	0.111 0.206	0.036 0.060	0.023 0.163	0.064 0.323	19.66 19.51	0.86 0.150	0.25 0.043	0.579 1.47	23.7 33.6	0.058 0.052	0.515 0.473	4.92 5.31
2 ^c	284 (5.0 kg)	Control ^d / Epinephrine	0.115 0.190	0.033 0.185	0.076 0.076	0.178 0.195	19.83 19.85	0.387 0.127	0.139 0.032	— —	25.8 65.1	0.062 0.040	0.499 0.488	4.75 5.31
3 ^c	286 (4.8 kg)	Control ^d / Epinephrine	0.100 0.245	0.045 0.128	0.055 0.037	0.065 0.152	19.28 24.97	0.252 0.287	0.061 0.068	0.031 1.55	5.58 34.0	0.052 0.060	0.482 0.514	4.68 5.68
4 ^c	286 (5.0 kg)	Control (av of Expts. 1 and 3) Epinephrine 30 min Epinephrine 60 min Epinephrine 120 min	0.105 0.146 0.274 0.285	— — — —	0.039 0.020 0.047 0.028	0.064 0.081 0.180 0.048	19.47 22.92 25.67 23.55	— — — —	— — — —	— — — —	14.6 35.6 38.0 31.9	— — — —	— — — —	4.80 5.07 5.87 6.49
5 ^{c,e}	286 (4.8 kg)	Control ^d (pH = 7.363) Epinephrine + 3% CO ₂ (pH = 7.356)	0.156 0.226	0.038 0.066	0.020 0.028	0.047 0.114	16.60 20.56	— 0.171	0.032 0.054	1.54 1.11	42.6 28.7	0.078 0.076	0.613 0.526	4.54 5.71
6 ^{c,f}	284 (5.0 kg)	Control ^d (pH = 7.470) Epinephrine + respirator (pH = 7.338)	0.100 0.266	Trace 0.097	0.007 0.013	0.017 0.045	24.04 24.11	0.315 0.302	0.024 0.038	— —	44.8 67.1	0.094 0.055	0.789 0.356	5.98 6.29
Means		Control Epinephrine (30 min)	0.116 0.213	0.038 0.107	0.036 0.083	0.074 0.151	19.88 21.98	0.453 0.216	0.101 0.047	0.717 1.377	28.50 44.02	0.068 0.056	0.580 0.471	4.97 5.56

^a Abbreviations: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; DPG, 2,3-diphosphoglycerate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; LACT, lactate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

^b All experiments except no. 4 have provided ATP and DPG data for Table I.

^c Anesthetized with Sernylan.

^d Anesthetized with Ketalar.

^e Anesthetized with pentobarbital.

^f Control blood drawn 9 days after epinephrine treatment and bleeding.

^g Control blood drawn 9 days before epinephrine treatment and bleeding.

^h Control (baseline) blood drawn 15 min before first epinephrine injection.

with 3.5 mg of epinephrine, we have a consistent and statistically significant ($P = 0.0144$) increase in monkey red cell ATP concentration. The average increase in red cell ATP was 19.5%. The effect which we have observed in the total adenine nucleotides is dominated by ATP increase. One possible explanation for this increase could be an increase in red cell glycolytic activity since ATP is a product of glycolysis. We think these increases are due to increased glycolysis and probably an increase in adenosine monophosphate concentration as well. *In vitro* measurement of glucose consumption or lactate production can be used to establish increased or decreased glycolytic rate. *In vivo*, however, it is not possible to measure glycolytic rate in a direct way, and the rate of glycolysis in blood as drawn may not reflect the true status.

However, a change of *in vivo* enzymatic activity may be detected by a change in the pattern of glycolytic intermediates. The mean concentration of each of the glycolytic intermediates after epinephrine injection expressed as a percentage of the mean control concentration is 184, 31, and 204 for G6P, F6P, FDP, and DHAP, respectively. Thus, on the average, these four intermediates have experienced about the same relative increase in concentration with the epinephrine injection. The twofold increases in the *in vivo* concentrations of rhesus red cell G6P, F6P, and DHAP, which we have observed subsequent to epinephrine injection, are comparable with an *in vivo* stimulation of hexokinase activity by the epinephrine or some substance derived from epinephrine or its physiological action. However, alternative interpretations need to be considered. Phosphofructokinase (PFK) is an important regulatory enzyme in glycolysis but we do not believe this enzyme is directly involved in the epinephrine injection experiments because, as stated above, the substrate (F6P) and its product (FDP) are both increased by about the same relative amounts. Stimulation of PFK would be expected to increase FDP and DHAP and decrease G6P and F6P relative to their respective control concentrations (or at least

increase the former two more than the latter two), but this was not found in our experiments. Also free ATP is an inhibitor of PFK (11) but if the increased ATP concentration subsequent to epinephrine injection was inhibiting PFK significantly, we should see decreased FDP and DHAP relative to G6P and F6P. Thus, inhibition of this regulatory enzyme due to increased red cell ATP concentration is apparently not occurring under these particular conditions. Therefore, our interpretation is that these changes in glycolytic intermediates and adenine nucleotides, subsequent to epinephrine injection, are most logically explained as the result of some kind of stimulatory effect on red cell hexokinase.

The concentrations of 3-phosphoglycerate and phosphoenolpyruvate, which are in the lower part of the glycolytic pathway, did not change in a consistent way. Since phosphate is required at the glyceraldehyde 3-phosphate dehydrogenase (GAPD) enzymatic reaction of glycolysis, it is conceivable that a variable decrease in blood phosphate concentration could be an explanation for the variable decrease in red cell 3-phosphoglycerate and phosphoenolpyruvate after epinephrine injection. We have not assayed for blood phosphate concentrations in any of our experiments and cannot eliminate the possibility that epinephrine might have induced certain body tissues to utilize phosphate more vigorously.

The GAPD reaction consumes nicotinamide adenine dinucleotide (NAD^+) and produces the reduced form of this cofactor (NADH). A decrease in the NAD^+/NADH ratio could presumably exert inhibition on the GAPD step (12). The NAD^+/NADH ratio can be decreased by an increase in the lactate/pyruvate ratio due to the near equilibrium condition at the lactate dehydrogenase enzymatic step (12). Blood lactate and pyruvate may come from various tissues as stated earlier. However, this source of GAPD inhibition cannot be logically invoked to explain our glycolytic intermediate pattern changes associated with epinephrine injection because the lactate/pyruvate ratio changes in the wrong direction (it decreases, which would tend to increase the NAD^+/NADH ratio). If the lactate/pyruvate ratio

were to have any significant bearing on our results, it should be to help rather than hinder GAPD *in vivo* activity. Since the glycolytic intermediate pattern changes we have found do not argue for GAPD stimulation, we conclude that changes in lactate, pyruvate, and their ratio probably have no significant bearing on our results.

The epinephrine treatment caused an apparent hyperventilation and, thus possibly, some decrease in blood CO₂ level and increase in blood pH. An increase in pH might be the cause of a glycolytic response. However, when the possible blood pH increase was prevented, either by ventilation with air and 3% CO₂, or by a respirator, epinephrine treatment led to essentially the same increases in concentration of red cell G6P, F6P, DHAP, and ATP as usually seen. Thus, hyperventilation does not appear to have been the cause of the increase in these metabolites.

There are at least two different theoretical mechanisms by which epinephrine might alter rhesus red cell glycolysis. Harrison and Gray (13, 14) have reported that various catecholamines at concentrations in the 10⁻⁵ to 10⁻³ M range stimulate yeast hexokinase and hexokinase from brain homogenates *in vitro* when Mg²⁺ concentration and pH were suboptimal and when the concentration of glucose exceeded the half-saturation level. The 3.5-mg dose that we injected into the monkeys would produce a calculated peak theoretical epinephrine concentration of 8.5×10^{-5} M in the plasma if the entire dose went immediately into the blood stream. If the epinephrine diffused into their red cells, the resulting monkey whole blood concentration would be 6.3×10^{-5} M. The analogous theoretical concentration associated with the 0.35-mg epinephrine dose, which did not change glycolytic intermediates or adenine nucleotides, would be one-tenth as great. Human erythrocytes have been reported to take up epinephrine (15) and various amines (16). Roston (15) observed rapid penetration of small amounts of epinephrine and norepinephrine into erythrocytes. If this penetration also occurs with rhesus red cells, the *in vivo* concentration of epinephrine inside the cell which is obtained may be more than the minimum required

for hexokinase stimulation and it might explain the altered levels of ATP and glycolytic intermediates in the epinephrine-injected monkeys.

An additional mechanism for glycolytic stimulation is suggested by the work of Rasmussen *et al.* (17). These authors have shown that rat red blood cells respond to isoproterenol with a propranolol-inhibitable increase in cellular cyclic adenosine monophosphate (cAMP) content. Epinephrine and isoproterenol at very low concentrations increased human red cell volume and rendered these cells much more subject to hypotonic hemolysis but did this without a demonstrable increase in cAMP content. This effect in human red cells did not occur in the absence of Ca²⁺ and these same authors have shown that isoproterenol reduces the exchangeable Ca²⁺ pool in human red cells. The data are consistent with a model in which Ca²⁺ rather than cAMP is the "second messenger" in the human red cell's response to epinephrine and isoproterenol. It is not known at this time whether cAMP or Ca²⁺ is more important as a second messenger for that system in rhesus red cells. In either case, several phosphoproteins known to be important in regulation in nucleated cells are both cAMP dependent (ultimately) and Ca²⁺ dependent. Some of these proteins exist in mammalian red cells and might have an effect on glycolysis.

In view of the permeability of red cells to epinephrine (15) and the documented direct effects of catecholamines on red cell size and osmotic fragility (17), it is our working hypothesis that epinephrine affects rhesus red cell glycolysis by acting directly on the erythrocyte. Further, we have found no logical mechanism by which the red cell glycolytic response could be secondary to an epinephrine response in other tissues. However, we can not rigorously exclude the possibility that the red cell response may be secondary in nature. We think this area deserves further study because it may shed additional light on the mechanism of catecholamine action.

Summary. Erythrocyte adenosine triphosphate (ATP) concentration was found to increase in adult rhesus monkeys which have been injected with 3.5 mg of epineph-

rine. The average increase in red cell ATP concentration was 19.5% (significant at the 1% probability level). Epinephrine injection also increased the concentration of the early red cell glycolytic intermediates, glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), and dihydroxyacetone phosphate (DHAP). Respiratory alkalosis, which might have been induced by the epinephrine, is not responsible for the glycolytic changes. This effect of epinephrine on rhesus red cell glycolysis is compatible with an *in vivo* stimulation of red cell hexokinase by epinephrine or some substance elicited by epinephrine and suggests the possibility that such a stimulation may have a physiologic role in the mammalian erythrocyte.

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Clinical and Laboratory Investigations of Monovalent and Combined Meningococcal Polysaccharide Vaccines, Groups A and C (39563)

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Highly purified group A and C meningococcal polysaccharide vaccines were developed at the Walter Reed Army Institute of Research, principally by Gotschlich, Arntstein, and Goldschneider (1-3). These vaccines have proved highly safe and effective (1-10) in studies in man, and vaccines of commercial source have been licensed for use in the United States and elsewhere.

The present report summarizes the clinical and laboratory findings in studies in adult subjects given one or more doses of single or combined meningococcal vaccines prepared in these laboratories. Additionally, data relating to stability of the vaccine on prolonged storage in dried or liquid form at various temperatures and to the use of thimerosal preservative in the diluent are presented.

Materials and methods. Vaccines. The vaccines were formulated in the Merck Sharp & Dohme Research Laboratories, West Point, Pa., employing purified polysaccharide antigens prepared by Dr. Thomas H. Stoudt and his associates of the same institute, but at the Rahway, N. J., site. The polysaccharides were assayed for their physical and biochemical attributes, and the final products were considered satisfactory for use in human beings after they passed the usual tests for release of product. All lots were in accord with the current standards under which these products are released for commercial distribution by the Bureau of Biologics, U.S. Food and Drug Administration. Each antigen was present

in a 50- μ g amount per 0.5-ml dose given sc.

Clinical studies. The subjects were persons in open populations in suburban Philadelphia or in the environs of San Jose, Costa Rica. Informed written consent was obtained. Blood samples were drawn immediately prior to and 2 or 4 weeks after a single dose of vaccine. Modified regimens were used when multiple vaccine doses were given. The patients were observed for local and systemic reactions 4 hr, and 1, 2, 3, and 10 days after vaccinations. Observations were made by physicians or nurses.

Bactericidal antibody assays. The sera were stored frozen at -20° until tests for homologous serum bactericidal antibody were performed using a modification of a procedure (11) employing adsorbed adult rabbit complement. A fourfold or greater increase in serum antibody titer following vaccination was considered to be significant. The lowest dilution of serum tested was 1:2.

Results. Monovalent and combined vaccines given in a single dose. Groups of 25 or 26 persons in the United States (Study No. 404) or 15 persons in Costa Rica (Study No. 400) were given a single dose of group A, C, or combined A-C meningococcal polysaccharide vaccine. Serum bactericidal antibody responses are given in Table I. The responses are expressed both as proportion of persons showing fourfold or greater increase in antibody titer and as geometric mean titer. The large majority of persons was without detectable antibody before vaccination. It is seen that almost all persons in the United States responded to vaccination with a fourfold or greater antibody increase, while 80% or more of the persons in Costa Rica showed such response. The significant finding was that there was no important dif-

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A, C, OR COMBINED A-C VACCINE.

Study no.	Vaccine given	Antibody response against																	
		Meningococcus A									Meningococcus C								
		Initial seropositive			Initial seronegative			Total group			Initial seropositive			Initial seronegative			Total group		
		$\geq 4 \times$	Rise	No./total ^a	%	G.M. ^b	No./total	%	G.M.	$\geq 4 \times$	Rise	No./total	%	G.M.	$\geq 4 \times$	Rise	No./total	%	G.M.
404 USA 19-62 years	Mono A (D365) Mono C (D367)	4/4	100	304	22/22	100	66	26/26	100	84									
19-62 years	Combo AC (D369)	5/5	100	97	20/20	100	64	25/25	100	71	4/4	100	152	22/22	100	80	26/26	100	88
400 Costa Rica 19-69 years	Mono A (D365) Mono C (D367) Combo AC (D369)	8/10	80	91	4/5	80	16	12/15	80	51	3/4	75	38	10/11	91	39	13/15	87	38
		2/3	67	161	11/12	92	45	13/15	87	58	3/3	100	64	11/12	92	29	14/15	93	34

^a Number showing rise/total number tested.

Geometric mean titer.

The antibody responses found in groups of persons given three different lots of combined meningococcus A and C vaccines in a further study are shown in Table II. The antibody responses to the combined vaccines were roughly the same as found in Study No. 404 above (see Table I). This showed the consistency of response obtained when different lots of vaccine were used.

The clinical reactions noted following administration of the single or combined meningococcal polysaccharide vaccines in Study No. 404 (Table I) are summarized in Table III. The reactions were limited to a mild febrile response in a few persons and to mild local reactions in the majority of subjects given vaccine containing group A antigen. Very few of the subjects showed a reaction following group C vaccine and there was no greater reaction in persons who received combined vaccine than in those given group A vaccine alone.

Multiple doses of vaccine. A small number of persons were given repeat injections of meningococcus A or C vaccine at 0, 1, and 6 months. Blood samples were taken prior to and 1, 3, and 7 months after the initial vaccination. The findings given in Table IV showed the anticipated antibody response to the first dose of vaccine, and there was no further response when an additional one or two doses were given subsequently.

Vaccine stability. It has been reported that lyophilized meningococcus A polysaccharide vaccine may be unstable on storage at temperatures above freezing (3) (cf. 12-14). Studies were undertaken in which the antibody responses in human subjects to freshly prepared lyophilized meningococcus A or C vaccines were compared with responses to the same vaccines stored at the temperatures for the time periods shown in Table V. There was no detectable decline in immunogenicity of meningococcus A vaccine stored for as long as 1 year at 4° or at -20°, or for meningococcus C vaccine

TABLE II. SERUM BACTERICIDAL ANTIBODY RESPONSES IN HUMAN SUBJECTS IN PENNSYLVANIA MEASURED 2 WEEKS AFTER A SINGLE DOSE OF ONE OF THREE LOTS OF COMBINED MENINGOCOCCUS A-C VACCINE (STUDY No. 412).

Lot no.	Antibody response against											
	Meningococcus A						Meningococcus C					
	Initial seropositive			Initial seronegative			Initial seropositive			Initial seronegative		
	≥4× Rise			≥4× Rise			≥4× Rise			≥4× Rise		
	No./total ^a	%	G.M. ^b	No./total	%	G.M.	No./total	%	G.M.	No./total	%	G.M.
C-D560	11/11	100	12 120	15/15	100	<2 147				25/26	96	<2 50
C-D561	4/4	100	9 147	21/22	95	<2 38	3/4	75	5 23	22/23	96	<2 57
C-D562	12/13	92	12 135	12/13	92	<2 61				25/26	96	<2 38

^a Number showing rise/total number tested.

^b Geometric mean titer.

TABLE III. CLINICAL FINDINGS AMONG HUMAN SUBJECTS WHO RECEIVED MENINGOCOCCUS A, C, OR COMBINED A-C VACCINE IN STUDIES CARRIED OUT IN PENNSYLVANIA (STUDY No. 404).

Clinical findings	Meningococcus vaccine ^a —No. positive at time indicated								
	Group A (26 persons)			Group C (25 persons)			Group A-C (26 persons)		
	4 hr	Day 1-3	Day 10	4 hr	Day 1-3	Day 10	4 hr	Day 1-3	Day 10
Local									
Erythema	5	8	0	0	0	0	5	7	0
Induration/swelling	4	4	0	0	0	0	2	5	0
Soreness	13	12	0	2	2	0	14	13	0
Maximum temperature (°)°F									
<99	22	21	22	22	23	23	23	22	19
99-100.9	4	4	0	3	2	0	3	3	1
101.9	0	1	0	0	0	0	0	0	0
Not taken	0	0	4	0	0	2	0	1	6

^a Same lots of vaccine as Fig. 1.

TABLE IV. SERUM BACTERICIDAL ANTIBODY RESPONSES IN HUMAN SUBJECTS 21-45 YEARS OF AGE FOLLOWING REPEAT INJECTIONS OF MENINGOCOCCAL GROUP A AND C VACCINES (STUDIES No. 329 AND No. 281, COSTA RICA).

Antibody following dose	Vaccine											
	Meningococcus A (C-A611)						Meningococcus C (C-A258-1)					
	Initial seropositive			Initial seronegative			Initial seropositive			Initial seronegative		
	≥4× Rise			≥4× Rise			≥4× Rise			≥4× Rise		
	No./total ^a	%	G.M. ^b	No./total	%	G.M.	No./total	%	G.M.	No./total	%	G.M.
0 ^c	—		10	—		<2	—		16	—		<2
1	7/7	100	105	2/2	—	23	0/1	—	32	7/9	78	12
2	6/6	100	64	2/2	—	23	0/1	—	16	6/9	67	6
3	6/6	100	72	2/2	—	23	0/1	—	16	6/8	75	6

^a Number showing rise/total number tested.

^b Geometric mean titer.

^c Vaccine given at 0, 1, and 6 months. Serologic tests on bloods taken at 0, 1, 3, and 7 months.

4° for 1½ years. It is not possible to obtain molecular weight data for the meningococcus vaccines used in these studies because the measurement, by K_d determination, was not evolved at the time the studies were done. Retrospective analysis indicates, however, that the K_d value for the A vaccine was about 0.45 and for C about

0.55. The A and C vaccines that were rehydrated with the thimerosal-containing diluent and stored at 4° for 14 days were found to be just as immunogenic as when rehydrated in physiological saline solution. Table VI shows that there was no significant difference either in fourfold or eightfold antibody titer rises or in geometric mean titers in persons given stored vaccine compared with the fresh product.

Meningococcal infections are an important cause for morbidity and mortality, especially during periods of epidemic resurgence. Monovalent polysaccharide vaccines against meningococcus A and C have been proved safe and efficacious in trials in man. The vaccines induce far less antibody in young children than in adults (1-10) but meningococcus A vaccine has been proved efficacious in babies 3 months of age or older (9, 10) and meningococcus C in children 2 years or older (5, 10), according to the best present evidence.

The administration of these two vaccines in combined form presents a substantial convenience and saving in costs for administration in areas of the world in which there is risk of both group A and C meningococcal diseases. It is of importance, as shown in the present study, that this can be accomplished without reduction in antibody response and

TABLE V. STABILITY ON STORAGE IN LYOPHILIZED FORM OF MENINGOCOCCUS A AND C VACCINES AS REVEALED IN TESTS FOR SERUM BACTERICIDAL ANTIBODY IN ADULT HUMAN SUBJECTS.

Storage		Homologous bactericidal antibody responses			
		$\geq 4 \times$ Rise		Geometric mean titer	
		No./total ^a	%	Prevaccine	Postvaccine ^b
Time	Temperature				
Fresh	—	27/29	93	4	82
1 year	4°	15/15	100	<2	67
1 year	-20°	13/13	100	<2	75
Fresh	—	44/46	96	<2	31
16 months	4°	44/47	94	<2	98

^a showing rise/total number tested.
^b 2 weeks after vaccination.

TABLE VI. STABILITY OF MENINGOCOCCUS A AND C VACCINE ON REHYDRATION WITH THIMEROSAL PRESERVATIVE SOLUTION AND STORAGE AT 4° FOR 2 WEEKS (STUDIES No. 388 AND No. 336).

		Homologous bactericidal antibody responses			
		$\geq 4 \times$ Rise		Geometric mean titer	
		No./total ^a	%	Prevaccine	Postvaccine ^b
Meningococcus vaccine	Treatment				
339	Thimerosal solution 2 weeks	27/27	100	2	94
339-23 to 339-26 years	Saline solution fresh	23/24	96	4	102
258-1	Thimerosal solution 2 weeks	27/29	93	2	54
258-1-22 to 258-1-26 years	Saline solution fresh	26/28	93	2	55

^a showing rise/total number tested.
^b 1 month after vaccination.

without increase in local or systemic reaction.

Serum bactericidal antibodies are presently considered to be the most meaningful indicators for protective immunity (15). The antibody responses to vaccination in adult persons, even in those without detectable antibody initially, are likely based on recall owing to previous experience with meningococcus A and C organisms or with agents bearing related antigens. It is not surprising, therefore, that second or third doses of vaccine given within a short period of time did not elevate the antibody titers above those obtained following the first dose of vaccine.

Lyophilized meningococcus A vaccine is commonly considered to be unstable on storage at 4° (3, 12-14). This was not found for the present material within the time period tested. Meningococcus A vaccine retained full immunogenic potency for 1 year and meningococcus C vaccine retained its potency for 1 1/3 years, the longest time periods tested. Brandt *et al.* (16) reported apparent instability of dried group A vaccine after storage at 4° for 2 years.

The groups A and C vaccines retained their immunogenicity when rehydrated in thimerosal preservative and stored for 2 weeks in the refrigerator. Stability on addition of thimerosal has also been observed by others (17). These are important attributes for the vaccines in their practical routine use.

Summary. Purified group A and C meningococcal polysaccharide vaccines prepared in these laboratories caused remarkably little local or systemic reaction and were highly effective in stimulating serum bactericidal antibody in studies carried out in adults. There was no measurable reduction in immunogenicity or increase in toxic reaction when the two vaccines were given in combined form in a single dose. Maximal antibody response was obtained following a single dose of vaccine and there was no increase in titer when additional doses were given 1 and 6 months later. Both vaccines retained their immunogenic potency on storage at 4° for at least 1 year and there was no reduction in immunogenicity on storage for up to 2 weeks at 4° following rehydration in thimerosal preservative solution.

The authors are greatly indebted to Joan Goshaw and Joan Staub for valuable technical assistance. We wish also to acknowledge the medical assistance of Drs. Frank D. Gray, George A. Starkweather, and W. Stuart Warren, and the nursing assistance of Janet Campbell, Karen Campbell, and Jane Laughhead. The administrators and personnel of the Haverford Township Schools are cited for their helpful cooperation.

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Term Persistence of Antibody following Enders' Original and More Attenuated Live Measles Virus Vaccine (39564)

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Enders' original live attenuated measles vaccine (1-3) was first licensed for use in 1963. Because of febrile and allergic reactions, human immune globulin containing measles virus antibody was most commonly administered with the vaccine (4). The more attenuated measles virus vaccine (Enders line, Attenuvax) (5) was introduced in 1968. This vaccine is made of attenuated virus and is given without human immune globulin.

Measles has been expressed (6-9) in recent years for the increased incidence of measles in children, some of whom were previously vaccinated. These real and putative vaccine failures have been found to have various causes, as will be discussed. The present report describes the persistence of measles antibody for 15 years in persons who received Enders' original measles vaccine with human immune globulin 8 years following Moraten line vaccine. Enders' original vaccine given without human immune globulin.

Materials and methods. Study 17 (2-4). Seronegative children, 1 to 7 years of age who resided in open populations in the Philadelphia area, were given at the same time but in opposite arms Enders' live attenuated measles virus vaccine (0.02 ml/lb body weight of human immune globulin). The vaccine was given on December 7, 1960, and October 31, 1961. A sample of subjects were bled at 0, 4 weeks, and approximately 15 months following vaccination. Sera for comparison purpose were also collected from vaccinated children who experienced natural

measles about the same time or within a few months following the time when the vaccine was given.

Study 97 (5). Groups of 258 to 273 initially seronegative children, residing in open populations in the Philadelphia suburbs, were given Enders' original Edmonston vaccine or the Moraten (Attenuvax) more attenuated measles vaccine during May and June of 1967. These children have been under continuing surveillance for persistence of antibody since that time. The present report concerns the findings with sera from a representative sample of children taken 1 month and 8 years after vaccination. All sera were stored frozen at -20° until tested. All selections of children in both studies were at random.

Antibody assay. The sera were titrated for content of measles hemagglutination-inhibiting (HI) antibody by a standardized procedure (10) employed here. Both serum samples for each subject were tested together in the same test.

Results. Figure 1 shows the persistence of measles antibody in persons who received the Enders' vaccine with human immune globulin. The geometric mean titers were 1:25, 1 month after vaccination, and 1:28, 15 years after vaccination. Decline or increase in antibody titers was shown in more than half of the individuals. Importantly, none of the subjects had become seronegative within the 15-year period. The pattern for antibody persistence was not remarkably different from that following natural measles, except that increase in antibody was more frequent in vaccinees, suggesting a higher rate for reinfection with measles virus in nature. The mean antibody titers following the natural disease were about 3.5 times higher than following vaccination.

The persistence of measles HI antibody 8

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years following vaccination with Enders' original and Moraten measles vaccines is shown in Fig. 2. Decreases and increases in antibody titer were evident in both groups due, probably in part, to loss of antibody and to reinfection with measles virus in nature. Only one of the antibody levels fell to zero value and this was in a recipient of Enders' original vaccine. The mean titers were retained remarkably well.

Discussion. The occurrence of measles in children who had been vaccinated has been recorded in several studies (6-9). Failures of vaccination have been found due to a number of contributory factors including (a) use of killed measles virus vaccine that af-

fords only short-term immunity; (b) the usual and expected failure of serologic response in 2 to 5% of persons who were given the vaccine under proper conditions; (c) the use of live measles vaccine of low or no potency owing to improper handling, storage temperature, overexposure to light, etc.; and (d) neutralization of the live virus in the vaccine by maternal measles antibody in infants and by administration of the vaccine concomitantly with human immune globulin in children less than 1 year of age.

The present studies show remarkable persistence of measles antibody for at least 15 years in all children who received Enders' original vaccine along with gamma globulin. All the children were at least 1 year of age when vaccinated and all had responded initially to vaccination.

Similarly, there was excellent retention of measles antibody for at least 8 years after Enders' original vaccine given alone or after Moraten live measles vaccine. Higher antibody levels were achieved initially following Enders' original vaccine, but this difference was small 8 years after the vaccine was given.

The sum of the evidence indicates that antibody against measles persists for long time periods, perhaps lifelong, following administration of vaccine of proper potency given to subjects at proper age. It would appear that the attributes of live vaccine itself are far less important in producing and maintaining immunity against the disease than the matters of proper handling and administration of the vaccine and in achiev-

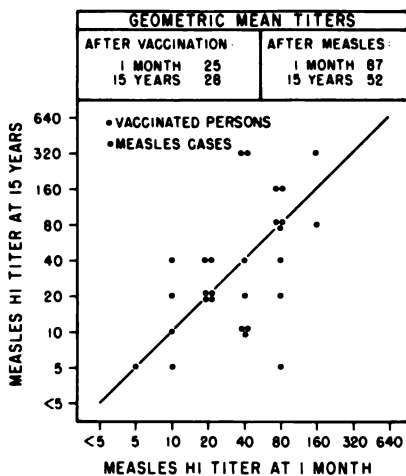


FIG. 1. Persistence of measles HI antibody for 15 years following administration of Enders' original measles vaccine coadministered with human immune globulin or following natural measles.

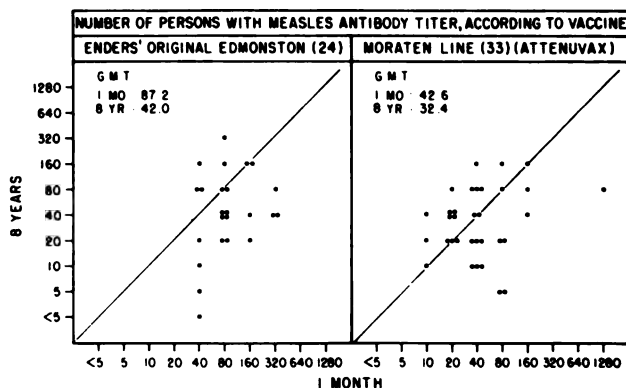


FIG. 2. Retention of measles HI antibody 8 years after vaccination with Enders' Edmonston or Moraten line measles virus vaccine.

ing maximal coverage of the susceptible population.

Summary. All of 20 children sampled who had received Enders' original measles vaccine, together with human immune globulin, retained measles antibody for 15 years with essentially no decline in antibody titer. All but one of 24 children who received Enders' original measles vaccine and all of 33 children who received more attenuated measles virus vaccine (Moraten line, Attenuvax) retained their antibody for 8 years, the longest time period tested. These data support the concept that the problems with efficacy of measles virus vaccine are more related to handling and administration of the vaccine itself than with the attributes of the vaccine.

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Tissue Culture of Rat Lenses¹ (39565)

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Sugar cataracts associated with diabetes and galactosemia have been studied extensively in animals (1-4). Numerous short-term experiments have also been conducted *in vitro*. A system which will permit long-term culture of lenses *in vitro* through the entire cataractogenic period, including the appearance of nuclear cataracts, is desired so that studies and correlations can be made under conditions that can be controlled and manipulated. In animals, the development of sugar cataracts is commonly studied in rats that are on a high galactose diet. A period of approximately 3 weeks is required. This paper describes, for the first time, a system that can be used to culture rat lenses for periods of 3 weeks and longer and permits galactose cataracts with morphological changes comparable to those found in rats to be produced and studied.

Methods. Apparatus. The apparatus is depicted in Fig. 1. The lens is supported on a perforated dialysis membrane (B), which is prepared by cutting it into single thickness squares about 3 × 3 cm, washing it for 1 hr in three to five changes of distilled water, making a depression to hold the lens in a stable position by stretching the wet membrane over the bottom of a 10 × 75-mm test tube and attaching it to the glass ring (C), securing it with a stainless steel wire, and perforating it with an 18-gauge stainless steel needle. A notch is cut in the membrane in line with the gaps in the glass ring and steel wire (E) and it is placed in a 15-ml beaker that has had the upper portion ground off so that it serves as the lower portion of a petri dish. The notches in the glass ring and perforations in the membrane permit components of the media to diffuse more freely and the notch in the membrane permits air bubbles to escape

from the under surface of the membrane. The notches and slit in the glass ring are made with a glass cutting wheel and the cover is made from the bottom of a 20-ml beaker. A drop of distilled water is added to the assembled unit (F) so that the membrane will remain moist while it is autoclaved for 20 min. Subsequently incubation medium is added and removed with pipets and suction using customary sterile procedures. This culture setup permits the lenses to be observed and measured with a low-power dissecting microscope and ocular micrometer.

Medium. The basic culture medium used for long-term incubation is a modified T.C. 199 (Gibco and Hanks' salts). It is fortified with additional buffer, magnesium, calcium, glutamine, fetal calf serum, and antibodies so that the final concentration (millimoles) of salts is 1.4 CaCl₂, 1.0 MgSO₄, 0.4 KH₂PO₄, 0.5 Na₂HPO₄, 10.4 NaHCO₃, 5.0 KCl, and approximately 112 NaCl. The medium contains 5.2 mM glucose, 13 mM glutamine, 100 units/ml of penicillin-G-potassium, 1 µg/ml of streptomycin sulfate, 2.5% fetal calf serum and the organic components of T.C. 199. Galactose and fructose may be added in the amount of 500 mg% for experimental and control media, respectively, if a cataractogenic medium is required. The concentration of NaCl may be varied to obtain the desired osmolarity, and the CO₂/air mixture of the incubator may be adjusted to the desired pH. The medium was sterilized by filtration through a 0.22-µm Millipore filter.

Incubation. Lenses are incubated in National CO₂/air-humidified incubators that are maintained in a special tissue culture room with a filtered positive-pressure air system and ultraviolet irradiation when the room is not in use. Surfaces are periodically washed with 1% Wescodyne solution to decrease contamination and the atmosphere within the incubators is continuously monitored with culture plates to be sure that the

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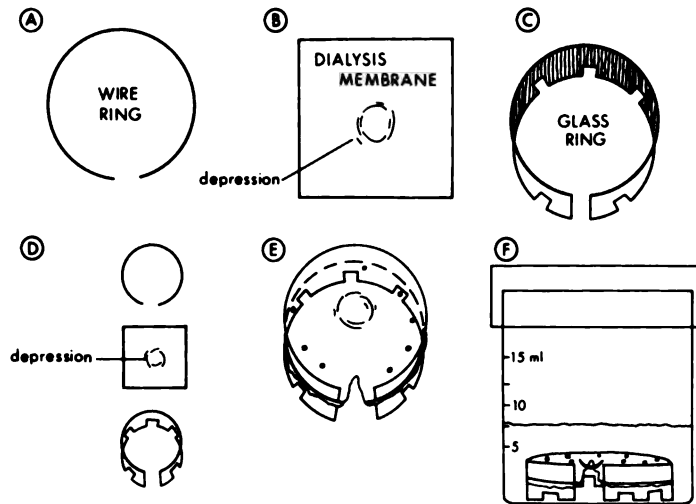


FIG. 1. Diagram of the culture flask with the membrane bed that supports a lens during incubation.

bility of contamination is minimal. Incubation conditions are considered to be ideal with a medium volume of 5 ml, an osmolarity of 295 mOsm, a pH of 7.3, and a temperature of 37°. For long-term cultures, medium is changed at 48-hr intervals.

Lenses. Lenses are obtained from young

Sprague-Dawley strain rats generally weighing 100 ± 10 g. Animals are sacrificed by decapitation, the whole eye is removed and put in a beaker containing 1% Wescobin in a 0.9% saline for 1 min. The eye is placed on a sterile towel and taken into tissue culture room where the lens is removed under sterile conditions under a hood. The adhering muscles are cut with a pair of straight scissors and held with forceps. A cruciform incision is made with a scalpel in the posterior surface extended with the aid of fine curved scissors and forceps. Care is taken to avoid going through the vitreous body. The eye is then transferred to a depression slide containing sterile media of the sort that is to be used in the experiment. The remaining dissection is done within the medium. The corneal opposite flaps are held with forceps, held apart, and inverted so that the tips of forceps come together over the cornea. The lens and vitreous body become free of the lens resting on the vitreous with its anterior surface exposed. The ciliary body is removed with very fine forceps and scissors. If the ciliary body is to be re-

tained, it is readily separated from the vitreous body with scissors. The lens is washed several times with sterile medium and transferred with the aid of a platinum scoop and wire loop to a culture dish. Similarly, following incubation, lenses are transferred with a loop to a piece of filter paper, rolled to remove excess media, weighed, and analyzed as desired.

Chemical. Sorbitol is determined as previously described (2).

Results. The results of incubating 27-mg lenses are shown in Fig. 2. The lenses were photographed with transmitted light so that opacities show as dark spots and transparent areas are white. Since the lenses of animals are usually observed with reflected light showing white opacities, the lenses are also shown after photographic reversal (2-B). The numbers indicate the days of incubation in standard medium (N) or galactose medium (G).

In a standard medium (N), lenses are transparent and normal in size at 23 days.

In a galactose medium (G), lenses go through the stages of cataract development that have been described for intact rats with nuclear cataract formation in 21 days (2, 4, 5, 6). In simplest terms, four stages may be recognized.

The first or early vacuolar stage involves changes in the equatorial region as seen in the photographs for Days 1-11. These changes are reversible *in vivo* (7).

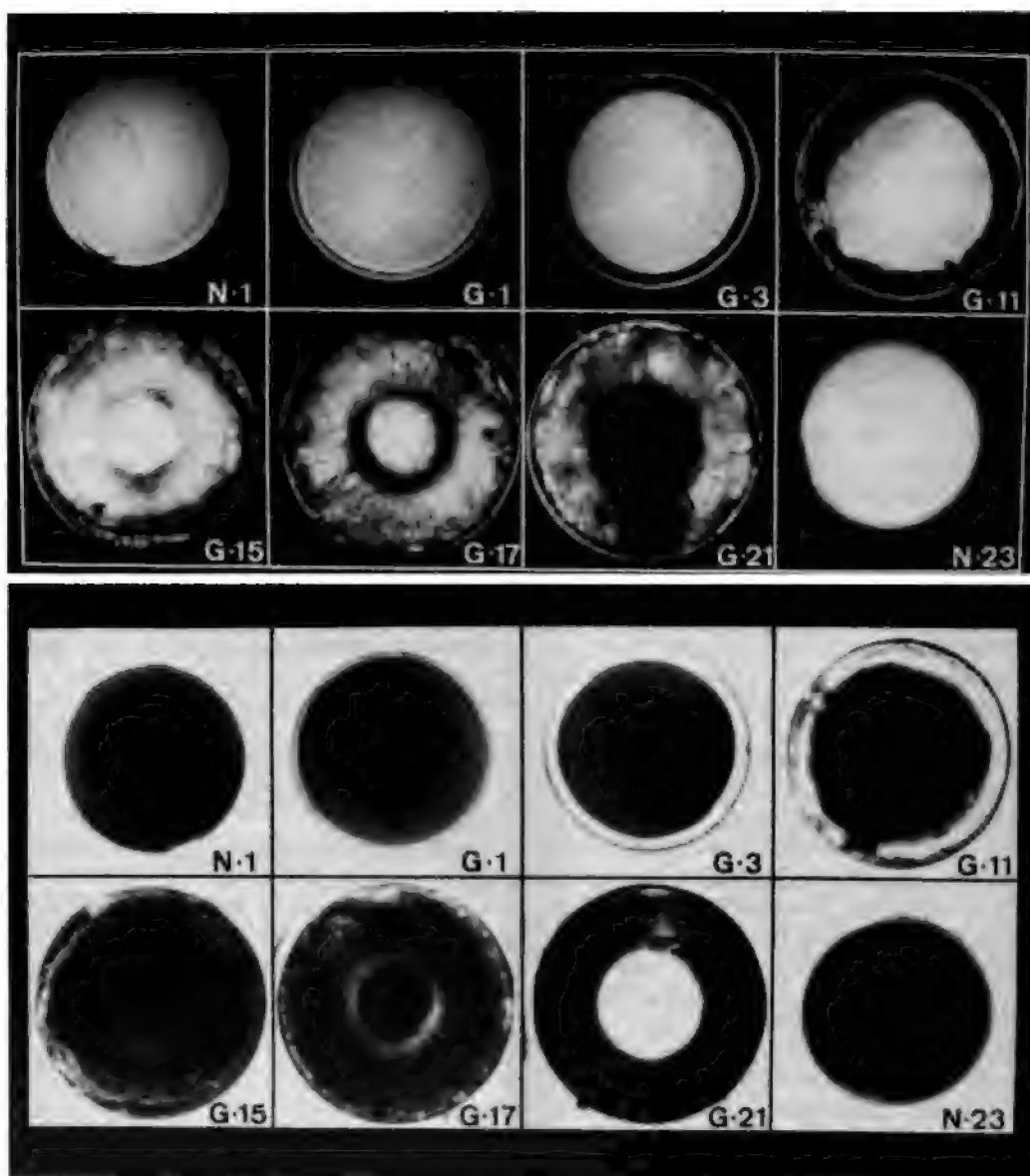


FIG. 2. (A) Direct photographs of lenses with transmitted light. The numbers indicate the days of incubation for lenses in standard medium (N) or galactose medium (G). (B) Photographic reversal to give the appearance of reflected light.

The second or late vacuolar stage is characterized by an extension of vacuolization to more central portions of the lens as seen for Days 15 and 17. At this stage the process is irreversible *in vivo* (7).

The third or nuclear cataract stage matures over a period of 24–36 hr and is easily recognized as a white nuclear opacity in re-

flected light and as a dark central area in transmitted light as in the photograph for Day 21. This relatively distinct and sudden change serves as an important end point in cataractogenic studies. This change is accompanied *in vivo* by a destruction of the lens fiber membranes (2).

The fourth or mature cataract stage is less

lite and consists of the gradual and complete opacification of the lens. It is observed after 25 to 30 days.

Retention of the ciliary body decreases trauma of dissection (8). This is noted in incubation of lenses in standard medium. Lenses with intact ciliary bodies could be incubated 30% longer (28 days) without bursting in size. Loss of transparency followed the onset of swelling.

Lenses incubated in galactose medium with dulcitol levels of 1100 mg/100 g at 2 with a resultant increase in size from 180 mm³ at 9 days (Table II). These results are in accord with observations in (3).

Discussion. The method that is described provides a means of studying lenses for transparency and size over a period of time without the necessity of sacrificing lenses. This will serve as a base for investigating cataract formation and other parameters of lens viability.

The presence of the ciliary body lengthens the time that lenses can be cultured without loss of size or transparency. The ciliary body is thought to limit swelling (Table II). The high proportion of burst lenses among those cultured in the galactose medium is thought to be due to this limitation. Lenses are known

to undergo rapid colloidal swelling following the third stage of cataractogenesis (3). If the ciliary body and intact capsule resist such swelling then enough pressure might develop so that the capsule will burst rather than accommodate to the pressure of gradual expansion. As indicated in Table I, the time at which lenses burst coincides with the appearance of nuclear cataracts in other lenses. This lends credibility to this explanation and in addition indicates that lens bursting should be equated with the appearance of nuclear cataracts as an endpoint in the study of cataractogenesis in tissue culture.

Summary. A system is described which

TABLE II. VOLUME OF LENSES INCUBATED IN GALACTOSE MEDIA.

Day	Without ciliary body		With ciliary body	
	Number	Volume \pm SE	Number	Volume \pm SE
1	7	18.5 \pm 0.7	7	17.9 \pm 0.6
2	7	20.5 \pm 1.2	7	19.5 \pm 1.0
5	7	23.6 \pm 1.4	7	22.6 \pm 1.0
7	7	27.2 \pm 1.4	7	25.0 \pm 1.5
9	7	30.9 \pm 1.6	4	29.6 \pm 1.8
12	7	29.6 \pm 1.5	3	22.6 \pm 2.2
14	7	28.4 \pm 1.0	2	28.4
16	7	27.2 \pm 0.9	2	28.4
19	7	23.6 \pm 1.4	1	24.8

TABLE I. DISTRIBUTION OF LENSES BY THE TIME REQUIRED FOR THE APPEARANCE OF NUCLEAR CATARACT IN GALACTOSE CULTURE MEDIUM.^a

Time of culture	Without ciliary body			With ciliary body		
	Cataract	Burst	Total	Cataract	Burst	Total
6	1	0	1	0	0	0
7	1	0	1	0	0	0
8	2	0	2	0	0	0
9	2	0	2	0	1	1
10	1	0	1	2	4	6
11	1	1	2	1	1	2
12	5	0	5	1	1	2
13	8	1	9	2	0	2
14	1	0	1	2	2	4
15	1	0	1	4	5	9
16	1	0	1	2	2	4
17	0	0	0	1	0	1
18	1	0	1	1	0	1
Number of lenses	25	2	27	16	16	32
Incubation time (day)	12	12	12	14.5	14	14
Incubation time (day)	11.8	12	11.8	14	12.9	13.5
	0.6	—	0.5	0.6	0.6	0.4

^aControl lenses without (8) and with (15) ciliary bodies incubated in standard medium were all clear and intact after 15 days.

permits rat lenses to be cultured and studied for periods in excess of 3 weeks without loss of transparency or an increase in size. When galactose is added to the medium, the lenses undergo morphological changes comparable with those found *in vivo* with the eventual production of nuclear cataracts. Lenses cultured with the ciliary body attached are less likely to be injured during dissection and can be cultured as effectively as isolated lenses.

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Serum IgA Levels and Antinuclear Antibody Formation in Mice¹ (39566)

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forms of autoimmune disease in humans are characterized by IgA deficiency and the appearance of antinuclear antibodies (3). By contrast, however, in aged mice the presence of autoantibodies is associated with an IgA hypergammaglobulinemia (4). This suggests that the relationship between the appearance of autoantibodies and deficiencies or elevations of serum IgA levels may be due to different mechanisms which may be age dependent. We have investigated mice for age-related changes in thymus and bone marrow function as they relate to serum IgA and the appearance of antideoxyribose protein (anti-DNP) antibodies in mice.

Materials and methods. A/J and DBA/1J mice were obtained from Jackson Laboratories (Bar Harbor, Me.) and were either 4-6 weeks of age (young mice) or 18-month-old breeders (old mice) when used. Only male mice were used in these experi-

ments. Thymectomy was performed in young mice using a standard suction technique under pentobarbital (Abbott Labs) anesthesia (5). Thymus grafts were obtained from 6-week-old (YBM) or 18-month-old (OT) syngeneic mice, irradiated, and placed on the cut surface of the recipient's kidney (6). All mice were examined for completeness of thymectomy and/or graft acceptance at autopsy both visually and histologically.

Representative tissue was taken from the site of thymectomy or thymus grafts. Sections were prepared using hema-

toxylin and eosin and examined microscopically. The data obtained from studies of animals with incomplete thymectomy or lack of graft acceptance are not included in these results.

Irradiation was performed using a ¹³⁷Cs source in a Model M Gammator (Radiation Research Corp., Parsippany, N.J.) at a dose rate of 110 R/0.1 min. Thymus grafts were irradiated to kill thymocytes at 550 R (7). Total body irradiation to induce IgA deficiency (8, 9) and destroy hemopoietic and lymphoid tissues was accomplished at a dose of 880 R. Irradiation was performed 1 week after thymectomy. Each mouse was irradiated individually in a perforated Plexiglas cylinder while rotating at 4.5 rpm.

Bone marrow cells were obtained from syngeneic 6-week-old (YBM) or 18-month-old (OBM) donors and diluted such that each recipient received 20×10^6 cells *iv*. Bone marrow reconstitution was performed within 2 hr following irradiation.

Blood was obtained from the periorbital sinus. Serum was separated and stored at -20° until testing for serum IgA or anti-DNP antibodies. Anti-DNP antibodies were determined by indirect immunofluorescence using undiluted serum (10). Each sample was independently evaluated by three observers and the average reading used (- or \pm readings were considered as negative). All other results are recorded as positive. DBA/1J mice were not tested for anti-DNP antibodies since they have previously been shown not to develop anti-DNP antibodies spontaneously (11).

Serum IgA levels were determined using radial immunodiffusion plates and standards (Meloy Labs).

Statistical tests were performed using Student's *t* test.

Results. Serum IgA levels increased with age in both A/J and DBA/1J mice in all test groups with one exception (Table I). The oldest DBA/1J group had lower IgA levels

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TABLE I. SERUM IgA LEVELS IN NORMAL A/J AND DBA/1J MICE.

Strain	Age in weeks	Mean IgA (mg/dl)	Range (mg/dl)	Number of mice tested
A/J	8	21	18-24	3
	12	34	18-66	10
	25	158	59-256	9
	80	212	70-367	9
	97	371	175-553	7
DBA	8	N.T. ^a		
	12	68	32-90	7
	25	146	82-195	10
	80	252	118-330	10
	97	193	128-256	6

^a N.T., Not tested.

than one of the younger DBA/1J test groups. In addition, the oldest DBA/1J mice had significantly lower IgA levels compared to the oldest A/J group ($P < 0.01$).

Thymectomy of young adult A/J mice had no significant effect on IgA levels at any age tested even though there was an accelerated appearance of anti-DNP antibodies by 21 weeks after treatment (Table II).

The reconstitution treatments varied considerably in their effectiveness to restore serum IgA levels to normal for age values and to prevent detectable anti-DNP antibody formation. Animals reconstituted with YT and YBM had normal or elevated IgA levels and normal anti-DNP antibody frequency at all ages tested.

In contrast, animals treated with OT and OBM failed to redevelop normal serum IgA levels, this being particularly noticeable at 21 weeks after treatment. These animals also had an accelerated appearance of anti-DNP antibodies.

Other experiments were performed using donor tissues of varying ages to determine whether the age of either the thymus, bone marrow, or both is the critical factor in reconstitution in terms of serum IgA levels and anti-DNP antibody. Animals grafted with OT and YBM had an increased frequency of anti-DNP antibody at 21 weeks after treatment. An initial high frequency of anti-DNP antibodies was observed in animals treated with YT and OBM but a reduction in frequency occurred by 21 weeks. The frequency at 21 weeks was still higher than normal but was lower than age-matched ani-

mals receiving OT and OBM or OT and YBM.

Reconstitution with YT and OBM was not followed by a recovery of normal for age IgA values. In contrast, animals receiving OT and YBM had normal or elevated IgA levels after reconstitution with the exception of those tested 21 weeks after treatment ($P < 0.025$). The critical factor determining the extent of recovery of IgA levels was the age of the bone marrow donor since both groups receiving OBM (OT and OBM; YT and OBM) failed to recover normal IgA levels by the end of the experiment. Conversely, both groups receiving YBM had normal or elevated IgA levels after reconstitution with the exception of the 21-week OT and YBM recipients previously noted.

TABLE II. SERUM IgA LEVELS AND ANTI-DNP ANTIBODY FREQUENCY IN A/J MICE AFTER ADULT THYMECTOMY, LETHAL IRRADIATION, AND RECONSTITUTION WITH THYMUS AND BONE MARROW FROM DONORS OF VARYING AGES.^a

Treatment	Number of weeks after treatment ^b	Mean IgA (mg/dl)	Anti-DNP antibody (% positive)
Young thymus, young bone marrow	4	22	0
	8	175	0
	21	168	0
Old thymus, old bone marrow	4	15	20
	8	19	80
	21	22	80
Young thymus, old bone marrow	4	20	50
	8	32	33
	21	18	20
Old thymus, young bone marrow	4	48	0
	8	57	0
	21	86	33
Thymectomized	4	N.T. ^c	N.T.
	8	52	0
	21	113	33
Normal young (no treatment)	4	21 ^d	0
	8	34	0
	21	158	0

^a Individual determinations were made in each test group, which consisted of four to six animals.

^b Treatment at 4-6 weeks of age.

^c N.T., Not tested.

^d Age-matched values from normal animals (Table I).

nectomy had no significant effect on levels at the ages tested in DBA/1J. The effects of the reconstitution treatment in this strain were not as pronounced in A/J mice (Table III). The YT and treatment was the most effective in raising IgA levels although both the 8- and 1-week treatment groups had lower values than normal animals. The values obtained with OT and OBM and those obtained with YT and OBM reconstitution were lower than normal values. Those animals receiving OT and YBM had nearly normal IgA levels 8 weeks after treatment, but 21 weeks after treatment the IgA levels were markedly lower than normal levels and were lower than those obtained in the YT and YBM group.

Discussion. Certain strains of mice such as NZB and NZM provide good experimental models of autoimmune disease. Anti-DNP antibody production in A/J mice is deter-

mined by age and genetic factors (10, 11) and is associated with a decrease in thymus-dependent immune functions with age (15-18). Thymectomy accelerates the appearance of anti-DNP antibody (15) and the formation of anti-DNP antibody can be reversed or delayed by treatment with thymus or spleen cells from young syngeneic donors (12, 19, 20).

Studies have also revealed that DBA/1J mice do not develop anti-DNP antibodies nor do they experience a decline in thymus-dependent immunity with age (11, 17). Such findings suggest that the thymus or T-dependent lymphocytes have a critical role in regulating the formation of anti-DNP antibodies (12, 15, 17). In addition, a loss of suppressor T-cell function occurs with age and this is a critical factor determining the appearance of spontaneous autoimmunity (12-14).

Our data suggest that the loss of thymic suppressor T-cell function may be involved in the appearance of anti-DNP antibodies in A/J mice. Irradiated young thymus grafts are effective in preventing or reducing anti-DNP antibody formation in young mice, but grafts from aged donors are not as effective. It is of particular importance to note that the irradiated thymus grafts we used consisted largely or entirely of thymic epithelial cells. There were significant differences in the anti-DNP antibody frequency in those animals receiving YT (YT and YBM) or OT (OT and YBM). Apparently, age-related involution of thymus function is reflected at least in part by changes in the thymic epithelial cells. It is significant that pathologic changes have been observed in thymic epithelial cells of New Zealand mice with autoimmune disease (21). In addition, thymic hormone preparations, which represent a humoral secretion of thymic epithelial cells (22), have been demonstrated by immunofluorescent techniques in thymic epithelial cells (23). A loss of thymic hormone function has been postulated as a significant cause of the immunologic abnormalities observed in New Zealand mice (24). Whether changes in thymic hormones are responsible for the age-dependent changes in thymus function in A/J mice remains to be determined.

Our data also suggest that a loss of sup-

TABLE III. SERUM IgA LEVELS IN DBA MICE ADULT THYMECTOMY, LETHAL IRRADIATION, RECONSTITUTION WITH THYMUS AND BONE MARROW FROM DONORS OF VARYING AGES.^a

Experiment	Number of weeks after treatment ^b	Mean IgA (mg/dl)	Range (mg/dl)
Thymus, bone marrow	8	48	29-87
	21	88	59-116
Thymus, bone marrow	8	34	29-41
	21	40	32-49
Thymus, bone marrow	8	37	21-52
	21	43	21-64
Thymus, bone marrow	8	61	52-74
	21	48	21-116
O-1	8	110	35-182
	21	178	52-256
Young control	8	68	32-90
	21	146	82-195

^a Individual determinations were made in each test which consisted of four to six animals. ^b Treatment at 4-6 weeks of age. ^c Unmatched values from Table I.

pressor cell function or potential to develop suppressor cells occurs within the bone marrow cell population with age. We have already noted that those mice receiving YT and YBM had normal for age anti-DNP antibody frequency. However, those mice receiving YT and OBM had increased anti-DNP antibody. Similarly, those receiving OT and YBM had a lower frequency of anti-DNP antibody when compared with those receiving OT and OBM. Since there are no suppressor cells in an irradiated thymus graft, the suppressor cell or its stem cell (or both) presumably originated from the donor bone marrow. This conclusion is supported by studies in which lethally irradiated mice given bone marrow from aged donors experienced impaired thymic regeneration capability (25).

The thymus has an important role in the establishment of normal serum IgA levels in mice. Several studies have indicated that there is a marked IgA deficiency in athymic nude mice in comparison to normal mouse strains (26-29). Treatment of nude mice which have a marked IgA deficiency with thymus cells or graft restores their IgA levels to nearly normal (28). Thymectomy had no significant effect on serum IgA levels in either A/J or DBA/1J which suggests that thymus function may not be as important in controlling IgA levels in aged animals as it is in younger or athymic mice.

Certain aged humans develop increased IgA levels associated with an increased frequency of autoantibodies and a decreased response of peripheral blood lymphocytes to PHA (4). IgA levels in the oldest A/J mice tested were elevated in comparison to DBA/1J mice of the same age and were associated with a high frequency of anti-DNP antibodies. In addition, previous studies have established that spleen cells of A/J mice develop a decreased response to PHA during aging (17, 18).

We observed that the age of the bone marrow donor was a critical factor in determining the effectiveness of our reconstitution procedures in terms of restoring IgA levels. Those animals which received bone marrow from aged donors had pronounced IgA deficiency at the end of the experiment. The reduced ability of bone marrow from

aged donors to reconstitute thymectomized, irradiated mice may be due to a relative inability of aged bone marrow cells to proliferate to generate functional B cells (30-33).

Summary. Aged A/J mice, an autoimmune strain, develop increasing levels of serum IgA compared to aged DBA/1J mice, a nonautoimmune strain.

Adult thymectomy did not affect IgA levels in A/J and DBA/1J mice but accelerated anti-DNP antibody formation in A/J mice. These results can be explained by spontaneous loss of suppressor T cells which prevent the formation of anti-DNP antibodies in A/J mice.

Experiments involving thymectomized, irradiated mice reconstituted with irradiated thymus and bone marrow from donors of varying ages suggested that there are intrinsic changes and thymic epithelium function with age. Bone marrow cells from aged donors were not as effective in restoring IgA levels and suppressing anti-DNP antibody as those from young donors.

Thymic epithelium from aged donors was not as effective as that from young donors in suppressing the formation of anti-DNP antibodies.

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Effect of Timolol on Hydralazine-Induced Increase in Plasma Renin Activity in Spontaneously Hypertensive and Normotensive Rats (39567)

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Timolol is a β -adrenergic-blocking agent which has been found to be relatively free of direct cardiac-depressant activity in animals (1, 2). Scriabine *et al.* (3) reported that timolol potentiated the antihypertensive response to hydralazine in spontaneously hypertensive rats (SHR). We studied the effects of timolol and propranolol on plasma renin activity (PRA) in SHR and normotensive rats. Antagonism of hydralazine-induced increases in PRA by single or multiple oral doses of timolol and propranolol was studied in SHR, normotensive Wistar (NWR), and normotensive Sprague-Dawley (NSDR) rats. We found considerable differences in the responses of various strains of rats to these β -adrenergic-blocking agents.

Methods. SHR of the Wistar-Okamoto strain were obtained from Charles River-Lakeview (Wilmington, Mass.). All rats were healthy females weighing 200 to 225 g. SHR were approximately 15-18 weeks of age while NWR and NSDR were approximately 12-15 weeks of age. All rats were allowed free access to food and water for at least 1 week prior to study and were fasted for 16 hr prior to oral drug administration. Drugs were dissolved in distilled water and administered by gavage (1 ml/kg body weight). Doses of all drugs were calculated in terms of the base. All rats were randomly divided into experimental groups.

All rats were sacrificed by decapitation. The initial 4 to 5 ml of trunk blood was collected in iced cups containing EDTA. PRA was estimated by radioimmunoassay for angiotensin I (A1) (Schwarz/Mann kit), and expressed as nanograms of A1 formed per milliliter of plasma per hour of incubation at 37°. All plasma samples from each study were assayed for PRA simultaneously

to reduce potential interassay variability. The experimental protocols were as follows.

Protocol No. 1. Groups of SHR were given single oral doses of (1) hydralazine,² 1 mg/kg; (2) timolol, 2 mg/kg; or (3) timolol, 2 mg/kg, followed by hydralazine, 1 mg/kg, 15 min later. A fourth group received 1 ml/kg of distilled water as a placebo. PRA was determined from plasma samples obtained 1 hr after administration of the drugs.

Protocol No. 2. Changes in PRA were determined in 15 groups of SHR after single oral doses of timolol, 2 mg/kg; propranolol,³ 10 mg/kg; or hydralazine, 4 mg/kg. Four rats were used in each group; five groups were used per treatment. The animals were sacrificed and blood samples for PRA assay were obtained at 0, 0.5, 1, 2, and 6 hr after drug administration.

Protocol No. 3. Dose-response studies with single oral doses of timolol or propranolol were conducted in SHR, NWR, and NSDR. Timolol was given at 0.5, 2, and 8 mg/kg, and propranolol at 2.5, 10, and 40 mg/kg. Four animals were used at each dose level of each drug. One group (four animals) of each strain of rats received distilled water as a placebo. The animals were sacrificed and blood samples were obtained 1 hr after drug administration. Geometric means \pm SE for PRA were calculated for each group.

Protocol No. 4. Groups of 9 or 10 SHR, NWR, and NSDR were treated orally with hydralazine, 4 mg/kg; timolol, 2 mg/kg; or hydralazine, 4 mg/kg, + timolol, 2 mg/kg, once daily for 4 consecutive days. In the last group, timolol was given 15 min prior to hydralazine. A fourth group received distilled water as a placebo. The animals were

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² Hydralazine was supplied by Dr. A. J. Plummer, Ciba-Geigy Corp., Summit, N. J.

³ Propranolol was purchased from Aldrich Chemical Co., Milwaukee, Wis.

iced and the blood samples were obtained at 1 hr after drug administration on fourth day of treatment.

Protocol No. 5. Groups of 10 NSDR were treated orally with hydralazine, 4 mg/kg/day; timolol, 10 mg/kg/day; propranolol, 10 mg/kg/day; timolol, 10 mg/kg/day + hydralazine, 4 mg/kg/day; or propranolol, 10 mg/kg/day + hydralazine, 4 mg/kg/day, for 4 consecutive days. β -Adrenergic-blocking agents were given 15 min prior to hydralazine in the last two groups. Two groups received distilled water as a placebo. All samples were obtained at 1 hr after administration and geometric means \pm SE for PRA were calculated for each group. Treatment-related changes in PRA were analyzed by a one-way analysis of variance procedure for each protocol.

Results. Protocol No. 1. PRA in 15 placebo-treated SHR was 2.7 ± 0.2 ng of A1/ml/hr (Table I).

In 16 SHR given hydralazine, PRA was 1.9 ± 0.2 ng of A1/ml/hr, which is significantly higher than in the placebo-treated group ($P < 0.02$). PRA in 16 SHR given timolol was 1.9 ± 0.2 ng of A1/ml/hr and significantly lower ($P < 0.05$) than in the placebo group. In 15 SHR given propranolol + hydralazine, PRA was not significantly different than in placebo-treated SHR (3.2 ± 0.5 ng of A1/ml/hr).

Protocol No. 2. In placebo-treated SHR, immediately after treatment was 1.7 ± 0.5 ng of A1/ml/hr. Timolol or propranolol produced equivalent reductions in PRA ($P < 0.05$) after 0.5 to 2 hr (Fig. 1).

Protocol No. 3. Peripheral PRA responses to graded, single oral doses of timolol or propranolol in SHR, NWR, and NDR are shown in Fig. 2. In SHR (Fig.

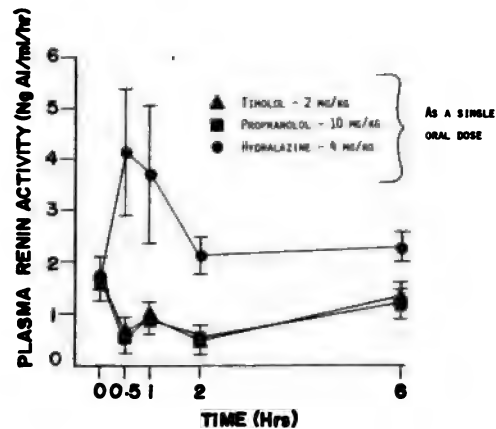


FIG. 1. Duration of action of timolol, propranolol, and hydralazine on PRA in spontaneously hypertensive rats. Each value represents the geometric mean \pm SE for a group of four rats.

2A), there was a negative, linear correlation between doses of β -adrenergic-blocking agents and PRA, and the slopes of regression lines were identical (see Fig. 2).

In NWR, timolol and propranolol at doses up to 10 mg/kg p.o. had no significant effect on PRA (Fig. 2B). Only at 40 mg/kg p.o., did propranolol lower PRA in NWR.

At the doses used, neither of the two β -adrenergic-blocking drugs had any dose-dependent effect on PRA in NSDR (Fig. 2C), although the PRA in rats treated with timolol was consistently lower than in the placebo-treated group.

Protocol No. 4. The results are summarized in Table II. There were no significant differences in PRA of placebo-treated rats from all three strains. These results support the findings of Sen *et al.* (4) that peripheral PRA in SHR is essentially the same as in NWR.

TABLE I. EFFECTS OF HYDRALAZINE AND TIMOLOL, BY SINGLE ORAL DOSES ON PRA IN SHR.

Exp no.	Treatment	Dose (mg/kg, p.o.)	Number of rats per group	Plasma renin activity (PRA) ^{a,b} (ng of A1/ml/hr \pm SE)
1	Distilled water, 1 ml/kg	—	15	2.7 ± 0.2
2	Hydralazine	1	16	3.9 ± 0.4
3	Timolol	2	16	1.9 ± 0.2
4	Timolol + Hydralazine	2 + 1	15	3.2 ± 0.5

^a A values are expressed as the geometric mean \pm SE.

^b Blood samples for PRA assay were obtained 1 hr after drug administration.

Hydralazine produced equivalent increases in PRA in all three rat strains. Timolol significantly lowered peripheral PRA in SHR ($P < 0.001$) and in NWR ($P < 0.001$), but not in NSDR. The combination

of timolol + hydralazine appeared to reduce PRA in SHR, but the effect did not reach a statistically significant level. In NSDR, PRA was significantly increased after timolol + hydralazine ($P < 0.001$). The comparison between rat strains revealed that after combined treatment with timolol + hydralazine, PRA in SHR was significantly lower than in NWR and NSDR, and PRA in NWR was significantly lower than in NSDR (both at $P < 0.05$).

PRA in hydralazine-treated rats was always significantly higher than with any other treatment schedule ($P < 0.001$) in any of the three rat strains used. Timolol antagonized hydralazine-induced increases in PRA in all three strains used. In SHR and NWR, timolol completely prevented the hydralazine-induced elevation in PRA.

Protocol No. 5. Two experiments were performed with this protocol in NSDR (Table III). PRA in two placebo groups was not statistically different. Likewise, PRA in the two hydralazine-treated groups was equally elevated.

Timolol or propranolol at 10 mg/kg/day significantly lowered PRA ($P < 0.05$ and $P < 0.01$, respectively). Timolol, 10 mg/kg/day, antagonized but did not prevent the hydralazine-induced elevation of PRA, while propranolol at the same dose prevented the hydralazine-induced elevation of PRA in NSDR.

Discussion. Peripheral PRA in the four groups of placebo-treated SHR was statistically equivalent and probably represents a range of normal PRA in active SHR subjected to daily handling and normal diet. Data from these rats also indicate that normal PRA in 200-g SHR is not significantly different from PRA in other strains of normotensive rats as was reported by Sen *et al.* (4).

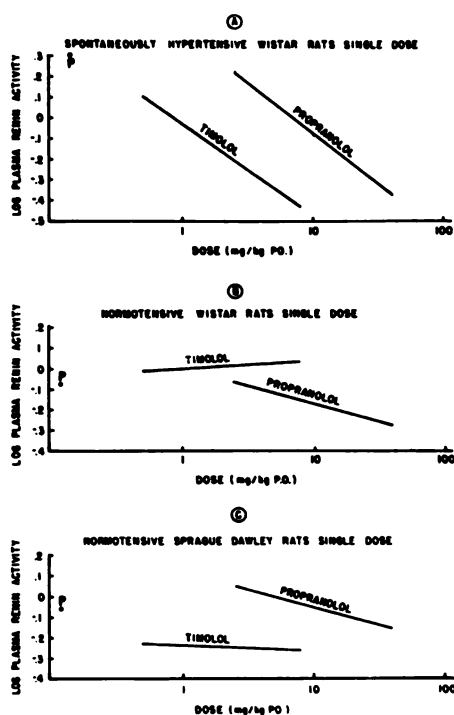


FIG. 2. Dose-response regression lines for the effects of timolol and propranolol on PRA in spontaneously hypertensive and normotensive rats. Timolol was given at 0.5, 2, and 8 mg/kg, p.o., and propranolol at 2.5, 10, and 40 mg/kg, p.o., to groups of four rats 1 hr before obtaining blood samples for PRA assay. Each value represents the geometric mean (expressed as log PRA) \pm SE for a group of four rats. \bullet Represents PRA in a group of placebo-treated rats of each strain. Slopes: (A) Timolol = -0.4455 ; propranolol = -0.4934 . (B) Timolol = -0.0418 ; propranolol = -0.1840 . (C) Timolol = -0.0206 ; propranolol = -0.1695 .

TABLE II. EFFECTS OF HYDRALAZINE AND TIMOLOL (DAILY FOR 4 DAYS) ON PLASMA RENIN ACTIVITY IN VARIOUS STRAINS OF RATS.^a

Strain of rats	Plasma renin activity (ng of Ang 1/ml/hr)			
	Distilled H ₂ O (1 ml/kg/day, p.o.)	Hydralazine (4 mg/kg/day, p.o.)	Timolol (2 mg/kg/day, p.o.)	Timolol + hydralazine
SH	2.0 \pm 0.4	7.0 \pm 2.0	0.5 \pm 0.1	0.9 \pm 0.3
NW	2.0 \pm 0.2	8.7 \pm 1.0	0.8 \pm 0.1	2.0 \pm 0.3
NSD	2.5 \pm 0.3	9.0 \pm 1.0	2.3 \pm 0.2	4.8 \pm 0.4

^a Geometric means for 9 to 10 rats per group \pm SE.

TABLE III. EFFECTS OF HYDRALAZINE AND β -ADRENERGIC-BLOCKING DRUGS (DAILY FOR 4 DAYS) ON ASMA RENIN ACTIVITY (PRA) IN NSD RATS.*

Treatment	Dose (mg/kg/day, p.o.)	PRA ^b (ng of Ang 1/ml/hr)	
		Expt. 1	Expt. 2
Water	—	2.5 \pm 0.3	2.1 \pm 0.2
Hydralazine	4	7.6 \pm 0.9	7.7 \pm 1.5
Timolol	10	1.6 \pm 0.3	
Timolol + hydralazine	10	5.7 \pm 0.7	
Propranolol	4		1.3 \pm 0.1
Timolol + propranolol	10		2.4 \pm 0.3
Hydralazine	4		

*ometric means for 10 rats per group \pm SE. ^bA was determined at 1 hr after treatment on the 4th day.

capitation can be used as a reliable method of collecting rat blood for PRA as provided only the initial 4–5 ml of blood are collected (5). Blood samples collected from rats under ether, halothane or barbiturate anesthesia are characterized by markedly elevated PRA (4–6). According to Scriabine *et al.* (3), the antihypertensive effect of single oral doses of hydralazine in SHR is potentiated by timolol. Timolol potentiates the antihypertensive effect of hydralazine. This has been reported also for propranolol in SHR (7). Hydralazine-induced peripheral vasodilation probably results from a reflex increase in PRA via secretion of adrenal catecholamines and/or via increased sympathetic nerve activity to renal juxtaglomerular cells (8, 9). By antagonizing the hydralazine-induced increase in PRA, timolol probably prevents subsequent secretion of angiotensin II. After timolol, hydralazine-induced relaxation of peripheral arterial smooth muscle was not blocked by circulating angiotensin II and, the antihypertensive response to hydralazine was enhanced. Timolol, 2 mg/kg, did not reduce resting arterial pressure, as reported by Scriabine *et al.* (3). These observations indicate that generation of angiotensin is not required for maintenance of hypertension in mature SHR, but that the responses to hydralazine-induced hypotension in SHR are angiotensin-dependent as in the NWR and NSDR.

Our experiments indicate that significant interstrain differences exist with regard to control of renin secretion in mature rats. In SHR, single oral doses of timolol and propranolol produce dose-dependent decreases of resting PRA (Fig. 2). The relative potency of timolol as a PRA-lowering agent in SHR was 7.7 times that of propranolol (95% confidence limits, 2.7 and 26.9 $g = 0.1975$). At the same doses, timolol did not reduce resting PRA in NWR, and propranolol was effective only at 40 mg/kg p.o. In NSDR, propranolol did not lower PRA even at 40 mg/kg p.o. and, at doses tested, timolol produced no dose-dependent effect on PRA. Because the regression lines for the effects of the two β -adrenergic-blocking agents on PRA in NWR and NSDR are invariant, the g -values are exceedingly high ($g = 3.4019$ and 2.0713 , respectively). This suggests that NWR and NSDR are relatively insensitive models for the evaluation of the effects of β -adrenergic-blocking drugs on PRA.

Striking interstrain differences in effects on PRA were also seen when hydralazine + timolol were given daily for 4 days (Table II). Resting PRA and hydralazine-induced increases of PRA were equivalent in all strains. There were significant interstrain differences with regard to the effect of timolol on resting PRA and prevention of hydralazine-induced elevations of PRA. In SHR and NWR, timolol reduced resting PRA and also prevented the hydralazine-induced increase in PRA. In NSDR, timolol neither lowered resting PRA nor totally prevented the hydralazine-induced increase in PRA.

Reduction of resting PRA in NSDR by timolol and propranolol at 10 mg/kg/day for 4 days (but not by 2 mg/kg/day) suggests dose-dependent variability among rat strains with respect to renin suppression by these β -adrenergic-blocking agents.

Results of these experiments indicate that timolol and propranolol suppress resting PRA and prevent the hydralazine-induced increase in PRA most effectively in SHR, as compared with NWR or NSDR. Timolol is more potent than propranolol in suppressing PRA in SHR.

Summary. The effects of two β -adrenergic receptor-blocking agents, timolol and

propranolol, on resting PRA and on the hydralazine-induced increase in PRA were studied in SHR and in two strains of normotensive rats [Wistar (NWR) and Sprague-Dawley (NSDR)]. With single oral doses, these β -adrenergic-blocking drugs reduced PRA in a dose-dependent manner in SHR but not in NWR or NSDR. After four daily doses, timolol antagonized the hydralazine-induced increase in PRA in SHR and lowered PRA in NWR. Timolol lowered PRA in NSDR only at 10 mg/kg/day p.o. for 4 days. It was concluded that SHR are more sensitive than normotensive rats to the PRA-lowering effects of β -adrenergic-blocking drugs.

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Range of Specific Activity of Plasma and Red Blood Cell Cholesterol following Intravenous Administration of [4-¹⁴C]Cholesterol in Man¹ (39568)

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Compartmental analysis of cholesterol requires the intravenously administered tracer cholesterol to be mixed instantaneously in the rapidly exchangeable pool sequentially to have the same metabolism as the unlabeled cholesterol (1). In all instances, the disappearance curve of plasma total cholesterol specific activity for compartmental analysis (2-9). The two major problems involved in this analysis are: (i) Cholesterol in the rapidly exchangeable pool exists in blood and varies in both free and esterified forms; (ii) complete mixing of the labeled cholesterol in this compartment cannot be instantaneous. Complete equilibrium between plasma and red blood cell free cholesterol is rapid as shown both *in vitro* and *in vivo* studies (10), whereas that between plasma-free esterified cholesterol can be achieved only after a longer time after giving [¹⁴C]acetate protein-bound [¹⁴C]cholesterol to the subject (11, 17) or [¹⁴C]acetate to man (13). Equilibrium cannot be attained by *in vitro* incubation of plasma because of the absence of the enzyme for hydrolysis of cholesterol esters (1, 14, 16).

Since cholesterol is virtually insoluble in water, the tracer cholesterol has been administered in the form of lipoprotein (17), in acetone, ethanol, saline, or ethanol-lipid emulsion (2-8, 19, 20), or as an emulsion emulsified with detergent (18, 21). The cholesterol specific activity during the first few days after administration has been shown to be greatly affected by the form of

tracer cholesterol administered. Cholesterol in the form of a suspension is rapidly removed from the bloodstream (18, 19, 21). There is direct evidence, at least in animals, that this is due to the uptake by phagocytic cells, especially the hepatic macrophages (19).

Ideally, the tracer cholesterol should be entirely in the form of lipoproteins. Methods for *in vitro* incorporation of cholesterol into serum lipoprotein have been described but require prolonged incubation (17, 18, 22) and have been adopted for human study by Sodhi and Kudchodkar (22, 23). However, the time for complete equilibrium between plasma-free and esterified cholesterol was not studied in their reports. Since the validity of the compartmental analysis depends upon the complete equilibrium of cholesterol in each pool, it is necessary to determine the time required for attaining such equilibrium, especially when the administered tracer cholesterol is not entirely in the form of lipoprotein. Such information is lacking in human studies and is the goal of our present research.

Materials and methods. Subjects. Two healthy adult white males, D. P. and K. B., measuring 169 and 175 cm and weighing 62.6 and 74.8 kg, respectively, were the volunteers for this study. Their plasma total cholesterol levels were, respectively, 208 and 199 mg/dl and free cholesterol levels were 52 and 56 mg/dl, as determined by the method of Sperry and Webb (24). Their serum lipoprotein electrophoretic patterns by agarose gel electrophoresis (25) were within normal limits.

Preparation and administration of the injectable [4-¹⁴C]cholesterol. Approximately 60 μ Ci of [4-¹⁴C]cholesterol in a stock solution containing 1 mCi in 6.8 mg of cholesterol (New England Nuclear, Boston,

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Mass.) was purified by silica-gel thin-layer chromatography in petroleum ether:ethyl ether:glacial acetic acid, 80:20:1 (by volume). Cholesterol was eluted with diethyl ether and sterilized by filtration (Millex 0.22- μ m filter, Millipore Corp., Bedford, Mass.) to two sterilized 10-ml test tubes in equal amounts and evaporated to dryness under a stream of nitrogen. Approximately 4 ml of fresh plasma obtained aseptically from each subject was added to the tube which was immediately incubated in a 37° water bath with constant shaking for 1 hr. At the end of 1-hr incubation, exactly 2.5 ml of the incubated plasma was injected intravenously back to the same subject. The remaining plasma was used for (a) routine bacterial culture, (b) immediate determination of the free and total cholesterol concentrations by the method of Sperry and Webb (24) and their radioactivities, (c) calculation of the total radioactivity administered to each subject, and (d) immediate agarose gel electrophoresis (25) for determination of the distribution of radioactivity among various classes of lipoproteins by cutting off the bands in the strip corresponding to chylomicron, β , pre- β , and α -lipoproteins and by counting their radioactivity in PPO-Bis-MSB toluene solution. The total radioactivities given to subjects D. P. and K. B. were 6.3×10^7 and 8.2×10^7 dpm, respectively. Six percent of [4- 14 C]-cholesterol was esterified after 1-hr incubation in subject D. P. and 9% in subject K. B. The distribution of relative radioactivity in the point of application, β , pre- β , and α -lipoprotein was 65, 29, 5, and 1% in subject D. P. and 62, 33, 4, and 1% in subject K. B. Apparently two-thirds of [4- 14 C]cholesterol was not incorporated into lipoproteins after 1-hr incubation and remained at the point of application during electrophoresis. The bacterial cultures were all negative.

Collection and analyses of sera. A 5-ml blood sample was collected from each subject in the following schedule: 12 min, 30 min, and 1, 2, 4, 8, 13.5, and 24 hr, and 3, 4, 5, and 7 days after the administration of [4- 14 C]cholesterol. RBC, after separation from plasma by centrifugation, were washed three times with 20 vol of normal saline. The cholesterol content of RBC was deter-

mined by the method previously described (26) and the serum-free and total cholesterol levels were determined by the method of Sperry and Webb (24). Their radioactivities were counted in PPO-Bis-MSB toluene solution. Quenching, when present, was corrected by the channels ratio method (27). Serum-esterified cholesterol concentration and radioactivity were calculated from the data obtained for free and total cholesterol.

Results. Changes of plasma and RBC cholesterol contents. The means and standard deviations of the 13 determinations of plasma and RBC cholesterol levels over a period of 7 days in subjects D. P. and K. B. were, respectively, 208 ± 6 and 199 ± 18 mg/dl for plasma total cholesterol, 156 ± 6 and 143 ± 12 mg/dl for plasma-esterified cholesterol, 52 ± 8 and 56 ± 5 mg/dl for plasma-free cholesterol, and 99 ± 6 and 97 ± 3 mg/dl for RBC cholesterol. Minor fluctuations were noted from time to time, as shown in Fig. 1.

Changes of cholesterol specific activity (Figs. 2-4). (i) *Plasma-free cholesterol specific activity.* The plasma-free cholesterol specific activity decreased sharply from the initial high value obtained 12 min after the injection in both subjects to a value only about 30% (28 and 31%) of the 12-min value in 2-4 hr. The specific activity then increased to a new high value 13.5 hr after injection. It then decreased again, steadily and gradually, to a value 10-16% of the initial high value on the seventh day.

(ii) *Plasma-esterified cholesterol specific*

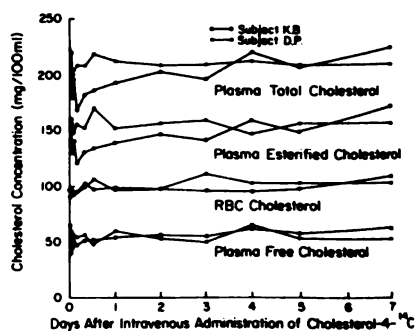


FIG. 1. Changes of plasma-free, esterified, total cholesterol levels, and red blood cell cholesterol contents in two subjects during the study.

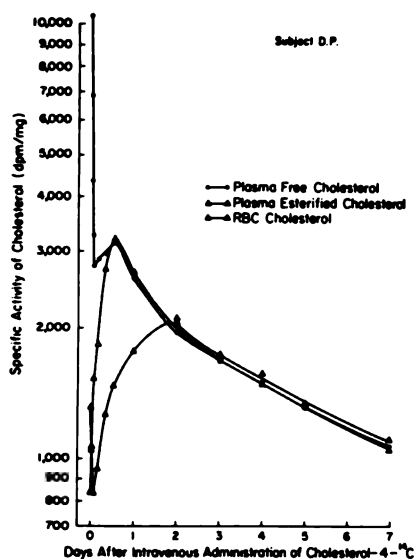


FIG. 2. Changes of the specific activity of plasma-free and esterified cholesterol, and red blood cell cholesterol after intravenous administration of $[4-^{14}\text{C}]$ cholesterol in subject D. P.

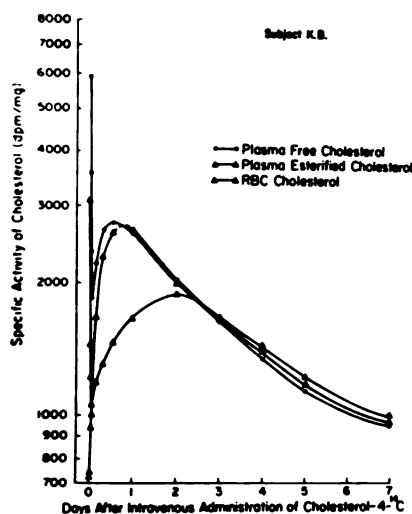


FIG. 3. Changes of the specific activity of plasma-free and esterified cholesterol, and red blood cell cholesterol after intravenous administration of $[4-^{14}\text{C}]$ cholesterol in subject K. B.

tivity. The plasma-esterified cholesterol specific activity obtained 12 min after injection was 52% of the plasma-free cholesterol specific activity in subject K. B. and only 15% in subject D. P. Nevertheless, it decreased sharply in both cases to a low value 2–4 hr and then increased gradually to

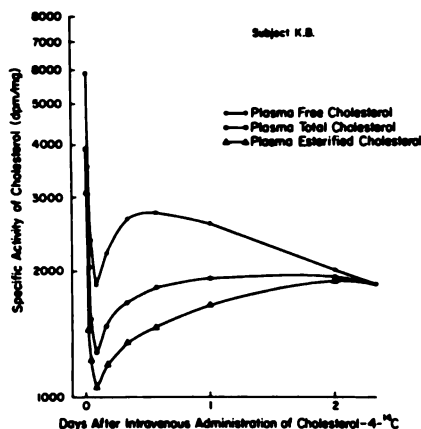


FIG. 4. Changes of the specific activity of plasma-free, esterified, and total cholesterol during the first 2 days after the intravenous administration of $[4-^{14}\text{C}]$ cholesterol in subject K. B.

reach a value similar to that of free cholesterol 2 days later. It then decreased again parallel to the decreasing curve of the plasma-free cholesterol specific activity, but the former usually had a higher value than the latter at any given time.

(iii) *Plasma total cholesterol specific activity.* The change of the plasma total cholesterol specific activity fell between that of the free and esterified cholesterol specific activity; an initial rapid decline was followed by a gradual increase to a peak 2 days after injection and then a gradual decrease.

(iv) *RBC cholesterol specific activity.* The RBC cholesterol was not labeled at the time of injection but the specific activity decreased rapidly to reach a value equal to the plasma-free cholesterol specific activity 12–18 hr after the injection. The RBC cholesterol specific activity then decreased following a curve parallel to that of the plasma-free cholesterol specific activity.

Discussion. The changes of the specific activity of plasma and RBC cholesterol after an intravenous administration of $[4-^{14}\text{C}]$ cholesterol followed a very similar pattern in these two subjects. The initial rapid decrease of the plasma-free, esterified, and total cholesterol specific activity over a period of 2–4 hr indicated the disappearance of some of the administered $[4-^{14}\text{C}]$ cholesterol from the plasma. Apparently some of the disappeared $[4-$

^{14}C]cholesterol was incorporated into RBC, but the uptake by RBC could only account for a small portion of the total loss. Therefore, these must be a net loss of $[4\text{-}^{14}\text{C}]$ cholesterol from the bloodstream within the first few hours after the injection. Two-thirds of the administered $[4\text{-}^{14}\text{C}]$ cholesterol was not incorporated into lipoproteins and existed in a particulate form which would be subject to phagocytosis by the reticuloendothelial system, particularly hepatic macrophages as shown in rats by Nilsson and Zilversmith (19). After such initial fall of specific activity, $[4\text{-}^{14}\text{C}]$ cholesterol started to reappear in the blood because plasma cholesterol specific activity increased to a peak 13.5 hr after injection. Obviously the uptake and release of the particulate cholesterol by reticuloendothelial system are rather rapid processes.

The exchange of free cholesterol between plasma and RBC was also very rapid. A complete equilibrium between the plasma-free cholesterol and RBC cholesterol was attained within 12–18 hr. It has been shown that the equilibrium between RBC and plasma-free cholesterol both *in vitro* and *in vivo* is closely approached in 4–8 hr (11–18). A detailed study of the *in vitro* quantitative change of free cholesterol between plasma and RBC by d'Hollander and Chevallier (16) revealed an exchange rate of 0.065 mg/hr/mg of blood and a turnover time of 9.2 hr for RBC cholesterol. The exchange required no energy derived from glucose metabolism (15). Because of such rapid and complete exchange, RBC and plasma-free cholesterol can be considered as in the same pool.

The exchange between plasma-free and esterified cholesterol was a slower process as compared with that between RBC and plasma-free cholesterol. We found in this experiment that 2 days were required for their complete equilibration in man. A similar result was obtained in dogs given lipoprotein-bound free $[^{14}\text{C}]$ cholesterol (17). This process requires the enzymes involving esterification of free cholesterol such as lecithin: cholesterol acyltransferase (28) and hydrolysis of esterified cholesterol such as cholesterol esterase (29). The exchange rate would depend upon the activities of these

enzymes. This is apparently the rate-limiting step for complete equilibration between the labeled and unlabeled cholesterol in the rapidly exchangeable pool.

The cholesterol-esterification activity seemed to be more active in subject K. B. than in D. P., because after 1-hr incubation with their plasma, 9% of $[4\text{-}^{14}\text{C}]$ cholesterol was esterified in K. B. whereas only 6% esterified in D. P. The initial serum-esterified cholesterol specific activity was also higher in K. B. than in D. P. However, the subsequent changes of serum-free and esterified cholesterol specific activity were quite similar in both subjects.

Although the technique for administration of tracer cholesterol for compartmental analysis described in this paper was not a perfect one because of the incomplete incorporation of $[4\text{-}^{14}\text{C}]$ cholesterol into lipoprotein, an initial phagocytosis of the particulate $[4\text{-}^{14}\text{C}]$ cholesterol, and 2-day requirement for complete equilibration between the plasma-free and esterified cholesterol, it was simple, rapid, and safe. Since the specific activity of plasma total cholesterol 24 hr after injection was underestimated, the compartmental analysis should be based on the plasma total cholesterol specific activity obtained 2 days after the injection.

Summary. The tracer $[4\text{-}^{14}\text{C}]$ cholesterol, incubated at 37° for 1 hr with the subject's own plasma, was administered intravenously to two subjects. The initial rapid decrease of plasma cholesterol specific activity indicated a net disappearance of $[4\text{-}^{14}\text{C}]$ cholesterol from the bloodstream, presumably due to phagocytosis of the particulate cholesterol by the reticuloendothelial system. The initially disappeared $[4\text{-}^{14}\text{C}]$ cholesterol quickly reappeared in the blood. Complete equilibrium between RBC and plasma-free cholesterol was attained within 12–18 hr, whereas the equilibrium between plasma-free and esterified cholesterol required 2 days. Therefore, the compartmental analysis should be based on the plasma total cholesterol specific activity obtained 2 days after the injection.

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Effects of Fructose and Other Dietary Carbohydrates on Plasma Glucose, Insulin, and Lipids in Genetically Obese (Ob/Ob) Mice (39569)

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While there is no general agreement about the effects of various dietary carbohydrates on lipid metabolism and cardiovascular disease in humans, some experimental studies have shown that hyperlipidemia can be produced in both man and in experimental animals, such as the rat, when fructose or sucrose is fed in comparison to feeding glucose or starch (1-3). A metabolic explanation for this effect has been that absorbed fructose is preferentially transported into the liver where it increases lipogenesis (4). In addition, fructose feeding can result in lower plasma glucose, a reduced insulin response, and a decreased lipoprotein lipase stimulation. However, the reported results are not consistent and vary with experimental conditions.

Because genetically obese mice show increased lipogenesis, hyperlipidemia, and abnormalities in carbohydrate metabolism even when fed usual laboratory diets (5), this animal model was used to investigate any additional effects of dietary fructose in comparison to other carbohydrates on these parameters.

Materials and methods. Male weanling mice of the mutant strain, C57BL/6J ob (Jackson Laboratories, Bar Harbor, Me.), were distributed into groups of 12 obese (genotype, ob/ob) and 12 nonobese (genotype, +/?) mice, caged separately and fed one of four experimental diets and water *ad libitum* for 24 weeks. Body weight and food intake data were recorded weekly.

The experimental diets contained either fructose, cornstarch, sucrose, or glucose as the carbohydrate source and had the following composition (grams per kilogram of diet): carbohydrate, 643; casein, 200; hydrogenated vegetable oil (Spry, Lever Brothers, New York, N.Y.), 100; salt mix (6), 50; vitamin mix, 5.0; and choline chloride, 2.0. The vitamin mix in glucose contained the following (per gram of mix): thiamin·HCl, 0.8 mg; riboflavin, 1.6 mg; pyri-

doxine·HCl, 0.8 mg; calcium pantothenate, 5.0 mg; nicotinamide, 8.0 mg; folic acid, 1.0 mg; biotin, 40 µg; cyanocobalamin, 20 µg; menaquinone, 20 µg; retinyl acetate, 6500 IU; ergocalciferol, 650 USP units; *dl*- α -tocopheryl acetate, 10 IU.

Fasting blood samples were taken from the orbital sinus biweekly during the 24-week experimental period. Because it was only possible to obtain about 200 µl of blood from these mice each time, determinations of plasma glucose (7) and immunoreactive insulin (Phadebus insulin test, Pharmacia, Piscataway, N.J.) were made in alternate samples starting after the mice had been fed the experimental diets 2 weeks, and plasma triglycerides (enzymatic method, Dow Diagnostics, Indianapolis, Ind.) and total cholesterol (8) were determined in alternate samples starting at 4 weeks. Statistical analyses were performed using Scheffe's method for *a posteriori* comparisons between groups (9).

Results. Dietary fructose reduced body weight gain in the obese but not in the non-obese mice in comparison to the other dietary carbohydrates (Fig. 1). After the obese mice had been fed the experimental diets for 4 weeks, the fructose-fed group gained significantly ($P < 0.01$) less, 6.8 ± 1.4 g (mean \pm SE), in comparison to 12.5 ± 1.3 g, 13.6 ± 0.9 g, or 13.1 ± 1.3 g for the starch-, sucrose-, or glucose-fed groups, respectively. However, there were no longer any significant differences in body weight or weight gain after 16 weeks. There were no significant differences in food intake between obese and nonobese mice fed any of the carbohydrates or between different time periods, although there was a trend toward lower intakes as growth rate decreased. The mean daily food intake was 3.56 ± 0.05 g for obese mice and 3.49 ± 0.08 g for non-obese mice for the entire experimental period.

Because our collected data showed no

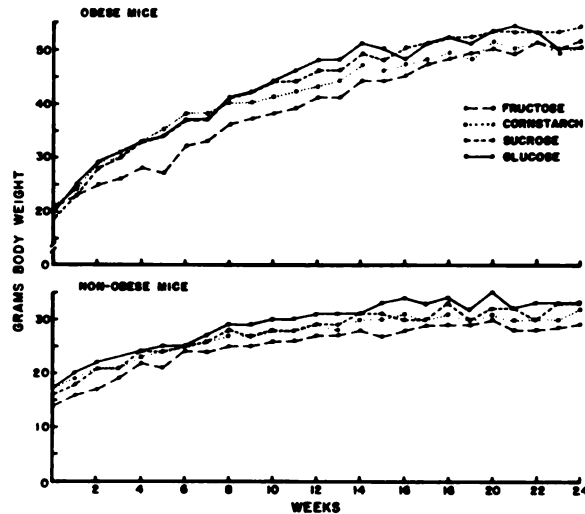


FIG. 1. The effect on body weight of feeding diets containing 64.3% fructose, cornstarch, sucrose, or glucose in obese and nonobese mice.

TABLE I. EFFECTS OF DIETARY CARBOHYDRATE ON PLASMA GLUCOSE, INSULIN, TRIGLYCERIDES, AND TOTAL CHOLESTEROL IN GENETICALLY OBESE AND NONOBESE MICE.^a

Dietary carbohydrate	Glucose (mg/100 ml)		Insulin (μ U/ml)		Triglycerides (mg/100 ml)		Cholesterol (mg/100 ml)	
	Obese	Nonobese	Obese	Non-obese	Obese	Nonobese	Obese	Non-obese
Fructose	88 \pm 8 ^{b, c} (44)	70 \pm 6 ^{b, d} (58)	62 \pm 9 (43)	14 \pm 2 (42)	108 \pm 11 (26)	86 \pm 9 ^b (41)	293 \pm 14 (33)	194 \pm 7 (37)
Cornstarch	128 \pm 8 ^c (42)	110 \pm 9 ^d (52)	80 \pm 13 (46)	15 \pm 1 (47)	139 \pm 17 (30)	147 \pm 18 ^b (36)	265 \pm 19 (26)	186 \pm 8 (41)
Sucrose	108 \pm 9 (47)	89 \pm 7 ^c (56)	62 \pm 9 (45)	14 \pm 2 (44)	113 \pm 14 (32)	124 \pm 12 (43)	333 \pm 17 (35)	212 \pm 8 (52)
Glucose	114 \pm 11 ^b (37)	123 \pm 8 ^{b, c} (61)	43 \pm 4 (40)	15 \pm 3 (49)	113 \pm 14 (27)	119 \pm 9 (48)	289 \pm 18 (23)	201 \pm 6 (53)

^a Each value represents the mean \pm SE. Numbers of samples are in parentheses.

^{b, c, d} For each column, the significant differences between groups are noted by the same superscripts: ^b $P < 0.01$; ^c $P < 0.05$; ^d $P < 0.01$.

change with time for any plasma parameter measured, the data were grouped for analyses as shown in Table I. The number of samples analyzed is indicated for each mean value; these numbers vary because some determinations were not possible due to inadequate blood volumes and early death of a few mice caused by the stress of repeated bleedings.

When comparing obese to nonobese mice, there were highly significant ($P < 0.001$) differences in plasma insulin and total cholesterol concentrations. While the kind of dietary carbohydrate had no significant effect on fasting insulin concentration, plasma glucose concentrations were affected

by feeding different carbohydrates to both obese and nonobese mice. Fructose-fed mice had the lowest plasma glucose concentrations. In obese mice, plasma glucose concentration was significantly lower in comparison to the starch- ($P < 0.05$) and the glucose-fed ($P < 0.01$) groups, but not the sucrose-fed group. In nonobese mice, the fructose diet produced significantly lower plasma glucose concentrations when compared to the means for the starch- ($P < 0.01$) and glucose-fed ($P < 0.01$) groups. Sucrose feeding also significantly ($P < 0.05$) reduced plasma glucose concentration in comparison to glucose feeding. Although the effect of the fructose diet was greater

than that of the sucrose diet in lowering plasma glucose, the difference was not significant.

Total cholesterol concentrations in plasma were similar for all carbohydrate-fed groups, and plasma triglyceride concentrations were also not affected by the type of carbohydrate fed, with one exception. Non-obese mice fed the fructose diet had a significantly ($P < 0.01$) lower plasma triglyceride concentration in comparison to starch-fed mice.

Discussion. Apparently, when fructose was fed to genetically obese mice as the only dietary carbohydrate, weight gain was not as great during the early postweaning period of rapid fat deposition, but the fructose-fed mice attained the same body weight as those mice fed starch, sucrose, and glucose as the growth rate slowed. Waterman *et al.* (10) have noted a similar depression in body weight gain as well as decreased food intake in rats fed fructose, in comparison to those fed glucose or sucrose. Our data did not show significantly lower food intake for the obese mice at the time when body weight gain was depressed. However, the food intake measurements were probably not accurate enough to detect the small difference in food intake which could account for the lower body weight.

The hyperglycemia usually observed in the genetically obese mouse was not observed in this study. This has been noted before in our laboratory in fasting obese mice fed semisynthetic, high-carbohydrate diets. The observation that sucrose feeding produced plasma glucose values between those of fructose and glucose or starch can be explained by the fructose part of the sucrose molecule. Since the genetically obese mouse can represent one model for human adult-onset diabetes, i.e., obesity associated with hyperinsulinemia, reduced glucose tolerance, and hyperglycemia, it is noteworthy that dietary fructose was beneficial in lowering plasma glucose under our conditions. However, if fructose is introduced into diets for diabetic humans, the amount would probably represent only a minor fraction of the total carbohydrate in the diet, and for this reason, may not have any effect on plasma glucose. While plasma

fructose concentration reportedly rises when rats are fed fructose (10), it is doubtful that any small increment in fructose concentration would affect total plasma hexose concentration in the fasting state. The fact that the fructose or sucrose diet did not raise plasma triglycerides in obese and nonobese mice is in contrast to that observed in man and the rat (1). Other investigators have shown that, at least in the rat, the sucrose or fructose effect on plasma triglycerides occurred either throughout life (11) or only in mature rats (12). Therefore, it is unlikely that we missed this effect in our study, and we must conclude that these mice reacted quite differently to dietary fructose by showing either no elevation or a reduction in plasma triglycerides when fed fructose in comparison to the other carbohydrates. Both the mouse and rat show high lipogenic activity in the liver (5) in comparison to peripheral tissues. Therefore, it is possible that either plasma clearance of triglycerides is more efficient in these mice or that lipogenic activity is not increased as much in the rat fed fructose diets. Our finding that the hyperlipidemia in the obese mice was due to hypercholesterolemia rather than to hypertriglyceridemia under our conditions demonstrates that this is a poor animal model for studying the effects of dietary fructose or sucrose in hypertriglyceridemia.

Summary. Genetically obese mice initially gained less body weight when fed diets containing fructose in comparison to mice fed starch, sucrose, or glucose diets. Plasma glucose concentrations were significantly lowered by fructose diets in both obese and nonobese mice in comparison to starch and glucose but not sucrose diets. Fructose or sucrose feeding did not induce hypertriglyceridemia in obese or nonobese mice as has been shown in the rat. In fact, the fructose diet significantly lowered plasma triglyceride concentrations in nonobese mice.

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Appearance of Inorganic Pyrophosphatase during Growth of *Streptococcus mutans* Cells¹ (39570)

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Inorganic pyrophosphate (PP_i), which is believed to be bound to the surface of hydroxyapatite crystals, has been suggested as a possible inhibitor of demineralization as well as a regulator of bone formation (1). Isolation and purification of inorganic pyrophosphatase (EC 3.6.1.1, PP_iase) from *Streptococcus mutans*, an organism highly implicated in dental caries (2), are the first phase in a study designed to determine if the enzyme, by hydrolysis of PP_i, is capable of initiating decalcification of enamel and dentin.

Before purifying a bacterial enzyme, two important questions must be answered. At what time during growth of the organism is the enzyme at its maximum level, and what cell disruption technique is appropriate for solubilization of the enzyme in terms of maximum yield? To answer these questions, an apparatus was designed to grow *S. mutans* cells which allowed continuous monitoring of the absorbance of the culture broth and the withdrawal of broth samples for analysis of cell PP_iase levels. Precise cell-harvest time for maximum yield of PP_iase as well as the appearance of PP_iase during growth of *S. mutans*, can be determined from use of the apparatus. Cell disruption techniques were also explored for an efficient method of solubilizing the enzyme.

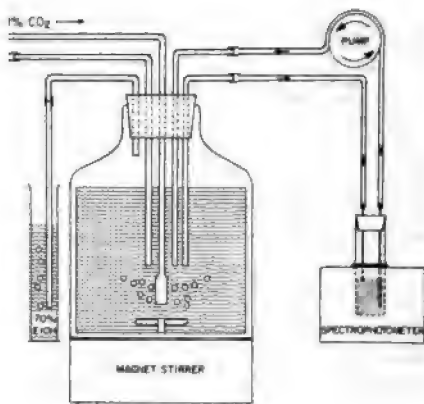
Methods. *Growth of S. mutans cells.* *S. mutans* K1R cells were grown in a 4-liter carboy (Fig. 1). Growth medium (4 liters) was 3% Todd-Hewitt broth (BBL, Cock-

eysville, Md.) and an additional 2% dextrose. The level of inorganic phosphate (Pi) in the broth was 40 mg/100 ml. Todd-Hewitt broth was autoclaved for 30 min, sterile dextrose was added, and all carboy connections were sealed or clamped before removal from the autoclave. Culture medium was inoculated with a 150-ml, 3% Todd-Hewitt, 16-hr culture of K1R cells and incubated at 37° while perfused with a mixture of 90% N₂-10% CO₂ to achieve microaerophilic conditions. A monostaltic pump circulated broth between the carboy and a cuvette tube in a Coleman Junior spectrophotometer (Coleman Electric Co., Maywood, Ill.) for a continuous monitoring of absorbance throughout the growth of K1R cells. Another line was installed from the broth to outside of the carboy to withdraw culture broth samples during the experiments of monitoring the appearance of PP_iase during growth of cells.

Growth of K1R cells was terminated at the height of log phase of growth by centrifugation at 10,000g, 20 min, and 4°. Harvested cells were washed three times with 0.05 M Tris-HCl, pH 7.5, and then resuspended, except as noted later, in 10 ml/g of cells (wet weight) of 0.1 M Tris-HCl, pH 8.0 (final cell-buffer suspension).

Appearance of PP_iase during growth of cells. At periodic times during the growth of cells, 50-ml broth samples, called periodic samples and labeled sequentially A-M, were removed from the culture and cells were immediately harvested by centrifugation at 10,000g, 15 min, and 4° (all following centrifugations were in this manner except as noted). Cell pellets from the periodic samples were individually washed three times with 0.05 M Tris-HCl, pH 7.5. PP_iase was solubilized from each periodic sample by suspension in 5 ml of 0.1 M Tris-HCl, pH 8.0, and ultrasonic treatment (described

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. Apparatus used to grow *S. mutans* K1R. 150-ml, 3% Todd-Hewitt, 16-hr growth of *S. mutans* was used to inoculate 4 liters of 3% Todd-Hewitt, supplemented with 2% dextrose; culture incubated at 37°. Stoppered line was used to draw growth samples as desired.

The periodic sonicates were also labeled with ^{32}P -ATP according to their originating cell sample and assayed for PP_iase activity.

Utilization of PP_iase. Five different disruption techniques were attempted to release K1R PP_iase from a complete cell suspension from 4 liters of culture growth. Cells were harvested at the height of the log phase growth on the basis of results from the assay of PP_iase during the 24-hr experiment.

Braun homogenization. Twenty milliliters of final cell-buffer suspension was added to 75-ml Duran flasks containing 30 g glass beads, 0.12-mm diameter. Flasks were shaken for 3 min in a Braun homogenizer (VWR Scientific, Baltimore, Md.) at 10,000 rpm under CO₂ flow for cooling. Glass beads were removed by filtration through a coarse sintered glass filter. Tris-HCl, pH 8.0, was used to rinse flasks and retained by the filter. Unbroken cells and cell fragments were removed from the filtrate by centrifugation.

French press. Final cell-buffer suspension was added to a precooled French press and forced through the flask at 15,000 psi (American Scientific Co., Silver Spring, Md.). Unbroken cells and fragments were removed from the homogenate by centrifugation.

Freeze-thaw. Final buffer suspension

was in 50 ml of 0.01 M Tris-HCl, pH 8.0. Cell suspension was frozen slowly overnight at -4°. Frozen cell suspension was thawed at room temperature and then centrifuged. Cell suspension was frozen and thawed five successive times in this manner. Supernatants were stored frozen until eventual pooling after the fifth thawing.

(iv) **Osmotic shock.** The cell pellet from the final cell wash was resuspended in 0.033 M Tris-HCl, pH 7.2, and osmotically shocked according to an established procedure (3).

(v) **Ultrasonic treatment.** Cells from the final buffer wash were resuspended in 25 ml of 0.2 M Tris-HCl, pH 8.0. This final cell suspension was sonified by a Branson sonifier, Model W185 (Heat Systems-Ultrasonics, Inc., Plainview, Long Island, N.Y.), with a flat sonifying tip at 4°, and a power setting between 8 and 9. After 15 min, cell suspension was centrifuged and supernatant pH was adjusted to 7.2 with 0.1 M Tris. Unbroken cell pellet was resuspended in 20 ml of 0.1 M Tris-HCl, pH 8.0, and sonified again as above. Third and fourth sonifications were in 10 and 8 ml, respectively, of the above buffer. Assays for PP_iase activity were accomplished in the individual homogenates and in a pool of homogenates from a 4-liter growth of K1R cells.

Assay of PP_iase activity. The PP_iase activity in the cell breakage supernatants was determined by modification of an established procedure (4). The assay solution, 1 ml and pH 9.0 (37°), contained 0.8 μmole Na₄P₂O₇ (PP_i), 1.6 μmole MgCl₂, 50 μmole Tris-maleate-Tris (Sigma Chemical Co., St. Louis, Mo.), and enzyme diluted in 2 μmole Tris buffer. Assay solution was incubated at 37° for 15 min. Enzyme activity was stopped by cooling to 4°. After 1 min of cooling, 2 ml of colorimetric solution of 0.75 N H₂SO₄, 0.375% (NH₄)₆Mo₇·4H₂O, 0.45% NaHSO₃, and 0.15% *p*-methylaminophenol sulfate was added to the standard assay solution. After an additional 5 min at 4°, assay-colorimetric solution was incubated at room temperature for 20 min and then the absorbance was determined at 670 nm. Maximum color development due to presence of phosphate, hydrolysis product of PP_i, was obtained by this procedure with insignificant acid hydrolysis of PP_i. Assays were done in

duplicate and a zero time enzyme blank, enzyme added after 15 min at 37°, was included with each duplicate. Known amounts of P_i -buffer tubes were also assayed as reference standards. A unit of PP_i ase activity is 1 μ mole of P_i released from PP_i per minute at 37°.

Protein determination. Protein concentrations were measured by the method of Lowry *et al.* (5). Bovine serum albumin was used as a reference standard.

Results. Formation of PP_i ase during growth of cells. Figure 2 shows the formation of PP_i ase activity by K1R cells during 24 hr of growth in Todd-Hewitt broth culture. PP_i ase activity increased rapidly during the early phases of growth. The rapid rise in activity was demonstrated by the activity curve coinciding with the growth curve until the height of the exponential phase of growth was reached, whereupon the PP_i ase activity fell about as rapidly as it rose during exponential growth. The enzyme activity leveled off at a base line level of activity of approximately 20% of the maximum reached at 0.5 OD units at 660 nm. The base line PP_i ase activity level continued throughout the stationary phase of growth. Periodic cell samples A-C required only 1-2 sonications each. Beginning with periodic cell sample D, the K1R cells were more difficult to disrupt. Periodic cell samples E-

M required 3-4 sonications for complete cell disruption.

Solubilization of *S. mutans* PP_i ase. Disruption of strain K1R cells by ultrasonic treatment, Braun homogenizer, French press, freeze-thaw, and osmotic shock methods revealed that ultrasonic treatment was the cell disruption technique of choice on the basis of measurable PP_i ase activity in the crude homogenates. Table I outlines the results of PP_i ase activity yield in the crude homogenates according to the disruption technique. Ultrasonic treatment of harvested K1R cells resulted in a yield of PP_i ase activity that was 280% of the PP_i ase yield from Braun homogenization of harvested K1R cells. Disruption by the French

TABLE I. DISRUPTION OF *Streptococcus mutans* STRAIN K1R CELLS BY FIVE CELL-DISRUPTION TECHNIQUES.

Cell disruption technique ^a	Units of PP_i ase activity	Protein (mg)	Specific activity
Ultrasonic treatment	4212	1266	3.3
Braun homogenizer	1540	1188	1.3
French press	98	121	0.8
Freeze-thaw	96	248	0.4
Osmotic shock	—	—	—

^a Each technique was applied to a 4-liter growth in Todd-Hewitt broth; cells were grown to OD of 0.5, at 660 nm.

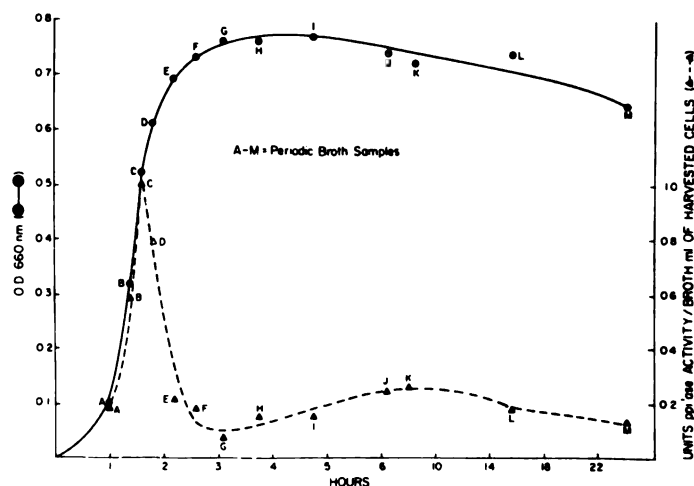


FIG. 2. Appearance of PP_i ase activity of *S. mutans* K1R cells during 24 hr of growth. At periodic times, A-M, broth samples were removed from the culture, centrifuged, and sonicated. The resultant periodic sonicates, A-M, were assayed for PP_i ase activity.

and freeze-thaw methods resulted in low levels of PP_iase activity. Osmotic shock of K1R cells yielded no detectable activity.

Fig. 3 depicts typical results of an acoustasonic treatment of harvested K1R from a 4-liter growth. PP_iase activity was greatest in the homogenate from acoustic treatment when cells are harvested promptly at 0.5 O.D. The pH of the acoustasonic homogenate was found to be as low as 4.8 when the K1R cells were harvested rapidly or when there was minimal lag phase of growth as shown in Fig. 2. It was found that the yields of PP_iase activity were always highest when K1R cells grew with minimal lag phase. Under such conditions, three ultrasonic treatments were sufficient for yield greater than 95% from cell disruption.

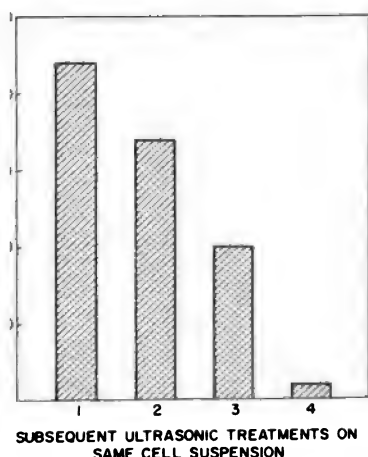
Discussion. The finding of the PP_iase activity rising rapidly to the same extent with increase in number of strain K1R cells cultured by optical density was similar to formation of phosphatase (P_iase) by *S. mutans* strain Ingbritt, cells (6). The activity of the strain Ingbritt cells remained at a relatively constant level after reaching its maximum level of activity. Contrary to this finding, the PP_iase activity of strain K1R cells fell about 80% immediately after reaching its highest level at the end of the log phase of growth. This re-

quires that the cells should be harvested at a precise point in the growth curve for maximum yield of PP_iase activity. The rapid increase in PP_iase activity can be explained by the organism producing the enzyme in parallel to the rapid growth of cells. The sudden fall in activity might be explained by curtailment of the production of PP_iase as cell growth is curtailed during the stationary phase of cell growth and the emergence of high levels of an inhibitor such as P_i.

High cell levels of P_i, as a product of PP_iase activity and as a result of growth in a phosphate-containing medium during this study, would be expected to inhibit the enzyme. As the cellular P_i level increases, production of additional P_i levels from PP_i may be inhibited by P_i binding to the enzyme molecule. Indeed, studies have shown cariogenic streptococci were able to concentrate intracellular P_i from tooth origin (7). A rapid drop in PP_iase activity on the part of *S. mutans* may be explained in part by the cell's ability to rapidly take in phosphate from teeth and the rest of the plaque environment. Also, the *S. mutans* PP_iase may in some way, yet to be determined, play a role in the cell's ability to tap P_i of teeth and alveolar bone supporting teeth by hydrolysis of PP_i originating from these structures. In so doing, hydroxyapatite of teeth and bone, according to the inhibitory role of PP_i (1), may be susceptible to demineralization as a result of biochemical activities by plaque microorganisms.

A demineralization-rem mineralization equilibrium has been postulated to take place at a potential carious site (8). During active caries, the equilibrium would shift toward demineralization. Additional recent evidence has suggested that alkaline PP_iase plays a role in the onset of calcification (9). Within the realm of the above postulation and according to the role suggested for alkaline and acid PP_iase (1, 9), *S. mutans* PP_iase may promote remineralization under alkaline conditions and demineralization under acid conditions.

Summary. It is important for the purpose of obtaining PP_iase activity to promptly harvest *S. mutans* K1R cells at the height of the log phase of growth or an OD of 0.5 at 660 nm. The apparatus shown in Fig. 1 enables



PP_iase activity in the supernatant of subsequent ultrasonic treatments on the same *S. mutans* suspension from a 4-liter culture growth.

one to grow streptococcal cells and to determine specifically during the growth cycle when to harvest the cells. Of the five cell disruption techniques tried, ultrasonic treatment is obviously the technique of choice for disruption of *S. mutans* K1R cells and solubilization of PP_iase for maximum yield.

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Stimulation of Leydig Cell Function in the Hypophysectomized Immature Rat (39571)

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mechanisms involved in the development of Leydig cell function during maturation of the testis are still unknown. Studies by Felföldi *et al.* (1) and Odell *et al.* (2) have shown that follicle-stimulating hormone (FSH) is a major factor in the development of testicular responsiveness to luteinizing hormone (LH) stimulation. These authors have shown that *in vivo* administration of FSH to immature rats induced testicular responsiveness to LH. When 21-day-old rats were hypophysectomized and 5 days later treated with FSH for 10, 25, or 30 days, plus LH during the last 5 days of therapy, an increase in testicular responsiveness to LH was observed. This increase in responsiveness, as measured by an increase in the weights of testes, was related to the duration of FSH treatment. The mechanism by which increased Leydig cell responsiveness has been ascertained. The present study was undertaken to elucidate the mechanism of action of FSH on Leydig cell function of immature hypophysectomized rat.

Materials and methods. Hypophysectomized Sprague-Dawley rats were obtained from Hormone Assay, Ltd. They were hypophysectomized at Day 20. All rats were started 5 days after hypophysectomy. Rats were injected subcutaneously twice with 20 μ g of NIH-FSH-S11, biological potency $1.15 \times$ NIH-FSH-S1 and less than $1 \times$ NIH-LH-S1, or with 1 μ g of NIH-LH-S19, biological potency $1 \times$ NIH-LH-S1 and less than $0.05 \times$ NIH-FSH-S1. Rats were given saline or the amount equivalent to that found in 20 μ g of FSH (2 μ g), twice daily. After 5 or 10 days of treatment, animals were killed and testes removed and weighed. One testis was used to determine the amount of LH

receptor and the other testis was used to measure testicular responsiveness to LH stimulation. Since it has been demonstrated previously that 125 I-hCG and 125 I-LH bind to the same receptor in a subcellular fraction of the rat testis (3), highly purified hCG (10,000 IU/mg) was labeled with 125 I by a chloramine-T technique as previously described (4) and used to quantitate testicular LH receptors. Testes were homogenized in 0.25 M sucrose buffered with 0.05 M HEPES, pH 7.4 (HS). The homogenate was centrifuged for 30 min at 20,000g. The 20,000g pellet was resuspended in 1 ml of HS. Aliquots of 0.1 ml were incubated in a total volume of 0.2 ml of HS containing 0.1% BSA, 5 mM CaCl_2 (HSB-Ca), and 100,000 cpm of 125 I-hCG ($\sim 60,000$ cpm/ng), and 100-fold excess of radioinert hCG in tubes in which nonspecific binding was determined. Incubations were carried out at 34° for 1.5 hr. Reactions were stopped by addition of 1 ml of ice-cold HSB-Ca. Hormone-bound membrane fractions were collected by centrifugation at 20,000g for 30 min. The supernatant was removed and the 20,000g membrane fraction was washed two more times with cold HSB-Ca. Radioactivity in each tube was determined by counting for 1 min in an automatic gamma spectrometer. Specific binding was calculated as the difference between binding in the presence and absence of excess radioinert hCG and expressed as counts per minute per testis.

To measure the effect of LH on testicular responsiveness, one-half of the contralateral testis was incubated in 1 ml of Krebs-Ringer buffer containing 2 mg/ml of glucose and 20 ng/ml of NIAMDD-Rat LH-I-3 (biological potency of $1 \times$ NIH-LH-S1 units/mg) and the other half of each testis was incubated in buffer without added rat LH. In a preliminary study, it was determined that 10 ng/ml

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of rat LH resulted in maximum testosterone production. Incubations were performed for 4 hr at 34° under 95% O₂-5% CO₂. To measure total testosterone, testes were homogenized in media; [³H]testosterone was added to monitor for losses and steroids were extracted with 75% ethanol, followed by two extractions with absolute ethanol. The dried ethanol extract was dissolved in 1 ml of phosphate-buffered saline containing 0.1% pig gelatin (gel PBS). The gel PBS solution was extracted with 10 vol of benzene:hexane (1:2). Each extract was dried and reconstituted in 1 ml of gel PBS. Aliquots of 0.1 ml were taken for counting [³H]testosterone and duplicate 50- and 200- μ l aliquots were used for radioimmunoassay. Testosterone was measured by radioimmunoassay employing testosterone-11 α -hemisuccinate-¹²⁵I-tyrosine methyl ester and a specific antiserum elicited in rabbits against testosterone-11 α -hemisuccinate conjugated to BSA (5). Testicular responsiveness to LH is expressed as the difference in testosterone production with and without added LH.

Results. Changes in testicular weight,

¹²⁵I-hCG binding, and *in vitro* testicular responsiveness are presented in Fig. 1A, B, and C, respectively. Testes from the saline-injected hypophysectomized rats regressed during the 10-day period of the study as indicated by a decrease in testicular weight, a decrease in LH receptors per testis, and a decrease in testosterone production in response to LH. Administration of LH even at five times the amount found in the administered FSH did not prevent the loss in testicular weight or the loss in LH receptors per testis, but appeared to maintain the capacity for testosterone synthesis in spite of the decrease in number of LH receptors. In contrast, FSH administration significantly stimulated all three parameters and the stimulation seen with FSH appeared dependent on the duration of FSH treatment. Percent increase in testicular weight and in LH-binding capacity were similar. Therefore number of LH receptors expressed on a weight basis did not increase with FSH treatment. However, the percentage increase in testicular responsiveness to LH was markedly greater than the percentage increase observed for testicular weight or LH receptors. Basal tes-

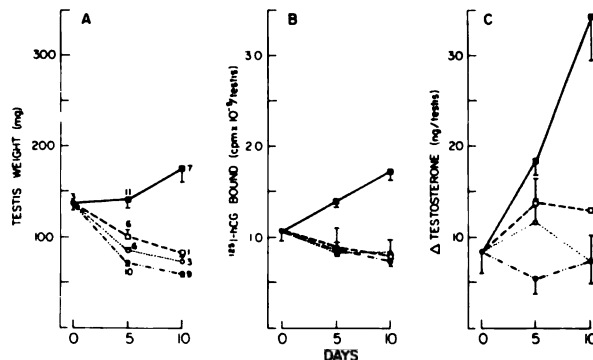


FIG. 1. Effects of hormone treatment on testes of hypophysectomized immature rats: saline (●), 0.4 μ g of LH/day (○), 2 μ g of LH/day (□), 40 μ g of FSH/day (■). Each point represents mean \pm SE. Numbers indicated at each point in (A) refer to all three graphs and represent number of rats. Student's *t* test was used to determine significant difference between means. (A) testicular weights in milligrams. $P < 0.001$ for all FSH-treated rats vs saline or 0.4- μ g LH control rats. (B) Amount of ¹²⁵I-hCG bound in counts per minute per testis. Aliquots of resuspended 20,000g testicular membrane fractions were incubated in buffer containing 100,000 cpm of ¹²⁵I-hCG for 1.5 hr at 34°. For determination of specifically bound ¹²⁵I-hCG, nonspecific binding, as determined by incubation of ¹²⁵I-hCG in the presence of 100-fold excess radioinert hCG, was subtracted from total bound counts. $P < 0.001$ for all FSH-treated rats vs saline or 0.4- μ g LH control rats. (C) *In vitro* testicular responsiveness to LH. One-half of each testis was incubated in 1 ml of buffer containing 20 ng/ml of rat LH and the other half of each testis was incubated in buffer without added rat LH. Incubations were performed for 4 hr at 34° under 95% O₂-5% CO₂. Total testosterone was measured by radioimmunoassay. $P < 0.001$ for FSH-treated rats vs saline controls; $P < 0.05$ for FSH-treated rats vs 0.4- μ g LH controls at 5 days; $P < 0.005$ for FSH-treated rats vs 0.4- μ g LH controls at 10 days.

ne production in testes from FSH- or ated rats was the same as that obtained in testes from saline-treated control hus, treatment with FSH increased ar responsiveness per LH receptor. ctions of LH-stimulated testosterone ion (Δ testosterone in nanograms :stis) to receptor ($\text{cpm} \times 10^{-3}$ G/testis) were 7.9, 13.2, and 20.0 at nd 10 days, respectively.

ussion. The present study demon- that FSH increased the number of eptors per testis. FSH was found to ite *in vitro* testicular testosterone pro- in response to LH. This LH-sensi- sticular response was enhanced to a extent than would be expected from rease in the number of LH receptors FSH treatment. These observations and extend studies recently cited by and Swerdloff (6). Thus, it seems un- hat the change in testicular sensitivity in the immature hypophysectomized be attributed solely to increased rs of LH receptors. The absence of a correlation between concentration of eptors and testicular response to LH en observed by other investigators. nd Dufau (7) added increasing con- ions of hCG to Leydig cell prepara- om adult testes, and observed maxi- stosterone production when fewer % of the LH receptors were occu- they concluded that Leydig cells con- large excess of LH receptors which necessary for eliciting maximal tes- ne synthesis.

ious studies by Means and Vaitukai- demonstrated that FSH binding was ed to cells of seminiferous tubules. ding of FSH to Leydig cells has been d to date, suggesting that the effect on Leydig cell function may be me- via seminiferous tubules. In prelimi- udies, we did not observe any ob- differences in Leydig cell histology n hematoxylin- and eosin-stained sec- f testes from saline- and FSH-treated he exact mechanism by which FSH ed Leydig cell responsiveness to LH s a matter of speculation. In this re- ve recently observed (5) that the *in*

vitro testicular capacity to synthesize testos- terone (measured as total testosterone pro- duction in the presence of LH) and the tes- ticular 17β -hydroxysteroid dehydrogenase activity follow the same developmental pat- tern. This observation suggests that 17β - hydroxysteroid dehydrogenase may be a limiting factor in the ability of the testis to respond to LH stimulation and that this en- zyme may be a key factor in the observed response to FSH treatment.

Summary. FSH treatment of hypophyse- ctomized immature rats increased testicular weight, increased the number of LH recep- tors per testis, and increased the *in vitro* testicular testosterone production in re- sponse to LH. The increase observed in all three parameters was related to the duration of FSH treatment. The testicular response to LH was enhanced to a greater extent than the number of LH receptors. The specificity of the FSH effect was indicated by the ob- servation that administration of LH had no effect on testicular weight or number of LH receptors. LH administration did not in- crease the *in vitro* testicular response to LH, but appeared to prevent the decrease in this response observed in saline-injected con- trols.

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Adipocyte Size Distribution in ob/ob Mice during Preobese and Obese Phases of Development¹ (39572)

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Adipose cell size and numbers in some strains of genetically obese rodents, such as the ob/ob mouse, have been shown to be elevated during development as well as in adult life (1, 2). These reports usually present only average cell size of the adipocytes along with an estimate of the total cell numbers in the fat body investigated or in the whole animal. The average cell size may misrepresent the development of adipose tissue because possible differences in the frequency distribution of cell size are ignored. Joosten and van der Kroon (3) examined cell size distributions in the ob/ob mouse. These authors only reported the cell size frequency distribution for three individual animals. Their presentation of data without formulation of a composite representation of several animals of particular phenotypes does not lend itself to statistical analyses.

In the genetically obese mouse (C57B1/6Jobob), adipocyte hyperplasia continues beyond the termination date for adipocyte proliferation, which is reported as 40 days for normal mice and 60 days for obese animals (1). This obesity segregates as a recessive trait and cannot visually be detected prior to 4-5 weeks of age (1, 4). Most workers have concentrated their efforts on the developmental stages after the phenotypic expression of obesity, due to the lack of an identifying test during the preobese phase. Many of their observations may be secondary to the obesity itself. Recently it was found that oxygen consumption of young

obese mice is considerably less than that of nonobese animals during the preobese phase of development (prior to 4-5 weeks of age) and can be used as a simple test for differentiation between future obese and nonobese animals (4, 5). Therefore, it is now possible to examine parameters of the obese state during the preobese phase of development.

In the present investigation, the frequency distributions of adipocyte cell size in the small- and moderate-size ranges are examined. The possibility is explored that differences in adipose cell size frequency distributions present during the preobese phase of development may be used to discriminate between future obese and nonobese animals. A method is also suggested for collection of individual cell size frequency distributions, with uneven numbers of total cells, into groups suitable for statistical comparisons.

Materials and methods. All animals used in the study were selected from the obese (C57B1/6Jobob) mouse colony at Michigan State University. The colony was originally derived from heterozygote (ob/+) breeding pairs that were purchased from the Jackson Laboratory at Bar Harbor, Me. The animals were raised in a temperature-controlled room at 25-27°, and had free access to food (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.) and water. The distribution of adipose cell size was studied during the preobese (less than 4 weeks of age) and obese (greater than 4 weeks of age) phases of development in ob/ob and non-ob/ob littermates. The ob/ob and non-ob/ob mice were identified at 3 and 4 weeks by the procedure of Kaplan and Leveille (4, 5), which uses a low oxygen consumption as a genetic marker for identification of animals genetically destined to become obese. Older animals could be visually distinguished as

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ob/ob or non-ob/ob.

mice were killed by decapitation at 3, 8, 10–12, and 16–18 weeks of age. After exsanguination, the epididymal fat of males and the parametrial fat bodies of females were dissected free. Duplicate samples were obtained from the older mice while only single tissue samples could be obtained from the younger mice. The tissues were immediately rinsed in distilled water, then in 0.9% NaCl, gently blotted dry, weighed, and fixed in 3% OsO_4 –50 mM collidine buffer, pH 7.4. The fixed adipose cells were gently isolated by serial filtration through a 40- μm sized nylon mesh by the method of Grollman and Gallian (6). Adipose cell size measurements were obtained on each tissue section with a Coulter electronic counter Model B equipped with Model J size distribution plotter). A 400- μm aperture was used. The instrument was calibrated with polystyrene latex grains. Cells were counted at 100V settings to determine the settings which minimized electrical noise in the system. Since we were interested in the development of smaller and moderate-sized cells, the diameter distribution between 40–92 μm was selected. The plotted frequencies of 25 categories on the distribution plotter were grouped into five size categories: 40–50, 51–60, 61–69, 70–80, and 81–92 μm .

The average cell size and cell size frequency distributions were calculated for individual animals. The average proportions for each size classification within a group were then determined as well as the average number of cells per sample that were counted. These proportions represent averaged data from four to six sample sections which contain an uneven number of total cells counted. This type of data does not lend itself to the use of the traditional chi-square to compare frequency distributions of one population with another. Therefore, a modified chi-square was performed. The average proportions for each classification were multiplied by the average number of cells per sample to obtain the average cell distribution in each size category that conformed to the average-calculated proportions. These average numbers were then used for the subsequent use of the chi-square test. Chi-

Results. The nonobese males exhibited an ordered distribution at 3 weeks of age (Fig. 1a). The largest proportion of cells was that with the smallest diameter counted, S_1 , and the smallest proportion of cells was that with the largest diameter counted, S_5 . Later in development, after 10–12 weeks, the order of the proportion of cells at each classification was reversed. The greatest proportion of cells was the largest cells counted, S_5 , and the smallest proportion of cells was the smallest cells counted, S_1 .

During the preobese phase of development, at 3 weeks of age, the ob/ob males (Fig. 1a) exhibited a frequency distribution different from that of nonobese mice. From high to low, the proportion of cells was that of size classifications S_4 , followed by S_3 , S_2 , S_5 , and S_1 . This distribution had a somewhat high proportion of larger cells during the preobese phase. In many respects, the order of size classifications exhibited by ob/ob males during the preobese phase was similar to that observed in the later stages of development among the nonobese males. After obesity was visually present, the proportions of the size classifications at later stages were radically different from the distributions observed during the early stages of development in both the obese and nonobese males. The order of proportions from high to low, was S_5 , S_1 , S_4 , S_2 , and S_3 during the obese phase. This is a bimodal distribution, with high proportions of large and small cells, a pattern quite different from that of the non-obese animals.

The nonobese females (Fig. 1b) exhibited a frequency distribution of adipocytes during development that was similar to that of nonobese males (Fig. 1a). Again, from high to low, the order of proportions at 3 weeks was S_1 , S_2 , S_3 , S_4 , and S_5 . The order was similar among the future obese females, but the proportions were significantly different from those of future nonobese animals (Fig. 1b, Table I). During the later stages, after 10–12 weeks, a bimodal frequency distribution of cell size was present among the obese females (Fig. 1b). High proportions of both large and small cells were present. As in the males, this bimodal distribution found

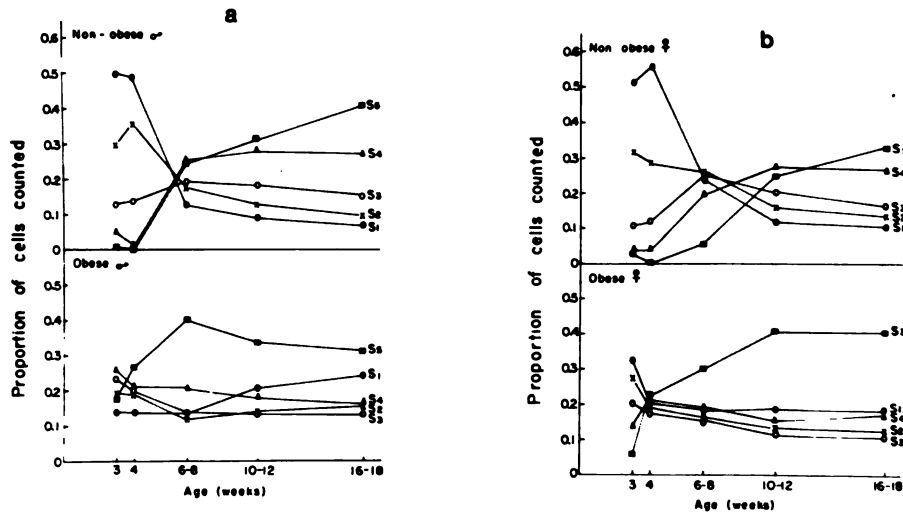


FIG. 1. Frequency of adipose cell size distribution during development in obese and nonobese mice. S_1 through S_5 refers to the diameter (micrometers) of cells in five size categories; S_1 (●—●) = 40–50 μm ; S_2 (×—×) = 51–60 μm ; S_3 (○—○) = 61–69 μm ; S_4 (Δ—Δ) = 70–80 μm ; S_5 (■—■) = 81–92 μm . Differences among the curves of obese and nonobese animals were evaluated by analysis of variance. In obese males, the curves of size classifications S_1 , S_2 , S_4 , and S_5 were significantly different from nonobese males with respect to age, phenotype, and age-phenotypic interaction at $P < 0.001$. Among the females, the curves of size classifications S_1 , S_2 , S_3 , S_4 , and S_5 were significantly different from nonobese females with respect to age at $P < 0.001$ or $P < 0.025$. With respect to age-phenotypic interaction among the obese females, the curves of S_1 , S_3 , S_4 , and S_5 were significantly different from the nonobese at $P < 0.025$ or $P < 0.001$.

among the obese females at 16–18 weeks was significantly different from the distribution among the nonobese females (Table I). A summary of the analysis of variance for two size classifications is presented in Table II. Only the smallest and largest sizes counted are presented as these are the most interesting. The analysis of variance indicates that the developmental patterns of both S_1 and S_5 class cell sizes are significantly different between obese and non-obese animals. It also indicates that the patterns significantly change throughout development. Both the chi-square test (Table I) and the analysis of variance (Table II) substantiate that the bimodal distributions observed among obese animals after 10 weeks of age are statistically significant. These bimodal distributions indicate that the number of small adipocytes has continued to increase in the obese animals throughout development as suspected by Johnson and Hirsch (1). Nonobese animals, in contrast, do not continue to proliferate adipocytes (1).

The bimodal frequency distribution of adipocytes among the obese animals would

have been completely undetected if we examined only the average cell size (Table III) from the preobese through the obese phases of development. In this situation, the average cell size was misleading. The increased number of both large and small cells in the distribution (Fig. 1a) would have been missed if only average cell size were examined. Also, during the later stages of development, at 10–12 and 16–18 weeks, the average cell size calculated for the obese males was smaller than that for the non-obese males (Table III). Among the female animals, no differences in average cell size were found at these ages. The bimodal distributions found in both obese males and females (Fig. 1) help to explain these average cell size findings at 10–18 weeks of age (Table III). Reliance on average cell size at later stages missed that the majority of cells among the obese animals was larger cells (Fig. 1). Concomitant increases in the proportion of small cells resulted in a decreased average cell size (Fig. 1; Table III).

We were confronted with the problem of how to conduct a test on proportions in order to determine whether the population

TABLE I. AVERAGE ADIPOCYTE POPULATION DISTRIBUTION IN OBESE AND NONOBESE MICE.^a

Age (weeks)	N	Phenotype	40-50 μm	51-60 μm	61-69 μm	70-80 μm	81-92 μm	Average number of cells per sample
Preobese phase								
3	6	Obese ♂	1079	1474	1766	1952	1343	7564
	6	Nonobese ♂	2408	1440	641	238	21	4817
	$\chi^2 = 3160, P < 0.001$							
	5	Obese ♀	1442	1225	908	621	273	4462
	5	Nonobese ♀	1825	1124	383	133	110	3563
	$\chi^2 = 555, P < 0.001$							
4	6	Obese ♂	1055	1428	1464	1514	2004	7476
	4	Nonobese ♂	3777	2776	1087	87	0	7723
	$\chi^2 = 5294, P < 0.001$							
	4	Obese ♀	1305	1247	1134	1346	1431	6469
	4	Nonobese ♀	5443	2778	1152	369	0	9764
	$\chi^2 = 4634, P < 0.001$							
Obese phase								
6-8	5	Obese ♂	249	214	244	372	722	1799
	4	Nonobese ♂	1050	1445	1631	2120	2018	8264
	$\chi^2 = 208, P < 0.001$							
	5	Obese ♀	441	387	372	460	711	2364
	5	Nonobese ♀	2026	2192	2108	1638	477	8426
	$\chi^2 = 1163, P < 0.001$							
10-12	4	Obese ♂	353	236	225	302	566	1680
	5	Nonobese ♂	827	1190	1683	2543	2824	9069
	$\chi^2 = 262, P < 0.001$							
	5	Obese ♀	418	296	253	343	899	2210
	6	Nonobese ♀	1328	1775	2265	3040	2776	11178
	$\chi^2 = 422, P < 0.001$							
16-18	4	Obese ♂	566	358	303	356	719	2300
	5	Nonobese ♂	588	839	1333	2333	3487	8577
	$\chi^2 = 743, P < 0.001$							
	5	Obese ♀	358	246	206	329	722	1910
	5	Nonobese ♀	752	958	1144	1872	2300	7028
	$\chi^2 = 188, P < 0.001$							

^a At each size classification, the average percentage of cells in Fig. 1 was multiplied by the average number of cells counted per sample for the group to obtain the values in the table. These calculated values conform to the average frequency distribution of each group. Each group was compiled from samples which contained markedly unequal numbers of adipocytes.

distributions at any particular age were different in obese and nonobese animals. The number of cells counted in each sample was very variable. The average number of cells within a size classification obtained from four to six samples did not represent the average proportion of cells at that classification. This would be true only if the number of cells in each sample were approximately the same. Therefore, to help reduce this problem, we multiplied the average propor-

tion of the samples at the particular size classification in question by the average number of total cells counted per sample to obtain the calculated average population distributions (Table I). From these numbers, a traditional chi-square was performed.

At every age studied, including the preobese phase of development, the adipocyte cell size distributions of the ob/ob mice were significantly different from the distri-

TABLE II. SUMMARY OF ANALYSIS OF VARIANCE FOR PROPORTIONS OF CELLS COUNTED AT TWO SIZE CLASSIFICATIONS.

Source	df	Mean squares	F	P > F
Males, Size 1				
Age	4	0.08110	16.60	0.001
Phenotype	1	0.10859	22.23	0.001
Age × phenotype	4	0.16870	34.54	0.001
Animal (age × phenotype)	41	0.00488		
Males, Size 5				
Age	4	0.15046	17.54	0.001
Phenotype	1	0.15024	17.52	0.001
Age × phenotype	4	0.04931	5.75	0.001
Animal (age × phenotype)	41	0.00857		
Females, Size 1				
Age	4	0.16046	23.32	0.001
Phenotype	1	0.07014	9.76	0.005
Age × phenotype	4	0.07730	10.75	0.001
Animal (age × phenotype)	39	0.00719		
Females, Size 5				
Age	4	0.18343	32.23	0.001
Phenotype	1	0.25138	44.18	0.001
Age × phenotype	4	0.02032	3.57	0.025
Animal (age × phenotype)	39	0.00569		

butions of the nonobese animals (Table I). The intraanimal variability was also fairly uniform throughout development for all size classifications studied (Table IV). Since the distributions at 3 weeks of age were extremely different for the future obese and future nonobese animals (Fig. 1; Table I), we hypothesized that the adipocyte cell size population distribution may be another useful way in which to discriminate between ob/ob and non-ob/ob animals during the preobese phase of development. To examine this hypothesis, a discriminant analysis (7) was performed with the aid of the Rutgers University computer. To discriminate between the ob/ob and non-ob/ob animals, the cell size distributions from the 3-week-old male and female mice were used. The resulting discriminant functions are shown in Table V. The discriminant function uses only four of the five adipocyte size classifica-

tions since the remaining fifth size classification is defined as a function of the remaining four. These discriminant functions (Table V) were then applied to each of the original animals at 3 weeks of age. The probability that each animal could be classified as either obese or nonobese was also calculated. All animals, but two obese females were correctly classified. The discriminant analysis suggests that the use of adipose cell size distribution may be another useful tool for differentiation of future obese and non-obese animals during the early preobese phase of development.

Discussion. Data were presented as composites of adipose cell size frequency distributions that represented four to six animals in a group. We also showed how such data could be manipulated so that they would be amenable to statistical analyses, such as a chi-square. Joosten and van der Kroon (3), in contrast, present adipose cell size frequency distributions for only three animals, with a statement that these are representative of the three genotypes, ob/ob, ob/+, and +/+. During the preobese phase of development, ob/ob and non-ob/ob individuals can be distinguished from one another on the basis of a lower oxygen consumption (5) as early as Day 17. It has also been suggested that ob/+ and +/+ individuals may also be distinguished on the basis of oxygen consumption values (5). Therefore, we would very much like to believe Joosten and van der Kroon (3) that these genotypes can be distinguished during the preobese phase on the basis of the adipocyte size frequency distributions. However, their presentation of data does not lend itself to statistical comparisons, such as chi-square or discriminant analysis. We also observed a sexual dimorphism in the frequency distributions at 3 weeks of age, which was not noted by Joosten and van der Kroon.

Johnson and Hirsch (1) observed that the total number of adipocytes increased until Day 60 in the ob/ob mice and to Day 40 in the non-ob/ob animals. They made the suggestion that this was due to an increase in the number of small adipocytes among the obese. Our data confirm this (Fig. 1). There was an increase in the frequency of small adipocytes throughout development which resulted in a bimodal frequency distribution

TABLE III. AVERAGE ADIPOSE CELL SIZE DURING DEVELOPMENT OF OBESE AND NONOBESE MICE.^a

Age (weeks)	Obese ♂	Nonobese ♂	Obese ♀	Nonobese ♀
Preobese phase				
3	67.4 ± 1.0 (6)	51.8 ± 1.8 ^b (6)	58.6 ± 2.0 (5)	52.1 ± 2.0 ^d (5)
4	68.7 ± 2.6 (6)	51.2 ± 1.0 ^b (4)	66.4 ± 1.4 (4)	50.6 ± 1.1 ^b (4)
Obese phase				
6-8	72.4 ± 0.4 (5)	67.8 ± 2.6 (4)	68.6 ± 0.6 (5)	61.1 ± 2.3 ^c (5)
10-12	68.9 ± 0.9 (4)	72.3 ± 0.7 ^c (5)	70.6 ± 1.1 (5)	69.9 ± 1.6 (6)
16-18	68.0 ± 1.6 (4)	75.1 ± 0.9 ^c (5)	70.8 ± 1.1 (5)	72.1 ± 2.1 (5)

^a Values shown are the means ± SE with the number of observations shown in parentheses. The average cell diameter was calculated as a grouped mean from the frequency distribution of each sample over the range 40-92 μ m. Preobese and obese phases refer to the developmental stages of genetically obese mice. Nonobese animals are matched by age to the obese animals.

^{b, c, d} $F > P$ at 0.001, 0.025, and 0.05 levels with respect to the appropriate obese group.

TABLE IV. INTRAANIMAL STANDARD DEVIATION.^a

	S_1	S_2	S_3	S_4	S_5	df
Males	0.0699	0.0515	0.0517	0.0373	0.0926	41
Females	0.0848	0.0533	0.0455	0.0473	0.0754	39

^a The intraanimal standard deviations at each size classification are derived from the mean squares obtained in the analysis of variance. The units are proportions as in Fig. 1 and Table II.

TABLE V. DISCRIMINANT FUNCTION COEFFICIENTS.^a

Adipocyte size class	Coefficient	Obese	Nonobese
Female			
1	b_{11}	447.83	457.87
2	b_{12}	638.75	660.09
4	b_{14}	920.59	910.17
5	b_{15}	267.72	288.01
Constant	b_{10}	-233.03	-243.53
Male			
1	b_{11}	728.93	742.08
2	b_{12}	1724.83	1826.80
4	b_{14}	1297.00	1238.33
5	b_{15}	942.69	940.05
Constant	b_{10}	-471.69	-491.84

^a The adipocyte size classifications have been defined in Fig. 1 and in the text. The discriminant function is defined as $y_i = b_{10} + b_{11}S_1 + b_{12}S_2 + b_{14}S_4 + b_{15}S_5$, where S is the proportion of adipocytes at the five size classifications as defined in Fig. 1 and in the text, and i = the phenotypic classifications; i = 1 or 2 for obese and nonobese, respectively. The probability that any animal can be classified as either obese (i = 1) or nonobese (i = 2) is defined as $p_i = e^{y_i}/(e^{y_1} + e^{y_2})$.

only among the obese group. Bergen *et al.* (8) observed that the total protein content of hind legs in ob/ob males was similar to that of non-ob/ob animals until Week 6.

After Week 6, the total protein content of the hind legs was considerably lower in the ob/ob than in the non-ob/ob animals. This approximates the date suggested by Johnson and Hirsch (1) for termination of adipocyte proliferation in the non-ob/ob animals. In the present investigation, we observed that the frequency of small adipocytes in the ob/ob males continued to increase after Week 6 (Fig. 1a). It can be hypothesized that adipocyte proliferation or recruitment occurred at the expense of lean or muscle growth in the leg preparation. Greenwood and Hirsch (9) suggest that preadipocytes may be formed until Day 35 in rats. Factors which regulate the differentiation of blast cells into preadipocytes or premuscle cells in the early neonatal period may be altered in ob/ob animals. This hypothesis has yet to be tested.

The adipocyte size frequency distributions were noted to be statistically different among ob/ob and non-ob/ob animals during the preobese phase of development. The results of the discriminant analysis suggest that the adipocyte size frequency distribution may be used for the early identification of ob/ob and non-ob/ob animals. A possible application to large domestic animals and

man may be made. The ob/ob and non-ob/ob animals were identified by the screening method of Kaplan and Leveille (4, 5), which uses the lower oxygen consumption as the identifying trait. The application of oxygen consumption as a screening device in large domestic animals and human children would be very cumbersome and expensive. Utilization of adipocyte size frequency distributions as a tool for early identification of potentially obese farm animals and children would be much easier. It would also not be necessary to determine the total body fat content to estimate the total adipocyte numbers for the animal. Only the adipocyte size frequency distribution would be necessary if it can be statistically compared by discriminant analysis to the frequency distributions obtained from known genotypes. This would be less expensive and faster than the determination of oxygen consumption in large animal chambers. Potentially obese meat animals could then be eliminated without wasting valuable feed grains on their growth. Potentially obese children could be carefully followed medically and guarded by parents to prevent a devastating childhood onset obesity which is difficult to treat.

Summary. The adipocyte size frequency distributions were determined during the preobese and obese phases of development in ob/ob and non-ob/ob mice. Only the small- and moderate-sized cells were evaluated. A procedure was presented that transformed composite data from the population

distributions of individual samples into a form suitable for comparisons by chi-square. Among the obese animals, the frequency of small cells continued to increase during development, which resulted in a bimodal distribution after 10 weeks of age. During the preobese phase of development at Week 3, the adipocyte size frequency distribution of the ob/ob mice was significantly different from that of non-ob/ob animals. Discriminant analysis suggested that adipocyte size frequency distributions may be used to identify ob/ob animals during the preobese phase of development.

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Effects of Chlorothiazide on Glucose Utilization, Glycogen Content, and Lactic Acid Production of Aorta¹ (39573)

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Benzothiadiazine drugs are widely utilized, both as diuretic agents and for the treatment of hypertension, but the cellular mechanisms responsible for these actions are not known. Thiazides also alter carbohydrate metabolism in both patients and experimental animals (1, 2). *In vitro* effects of chlorothiazide on glucose utilization in fat and liver have been demonstrated (3, 4). Similarly, the rate of utilization of glucose by dog aorta is reduced when chlorothiazide is added to the incubating solution (5). When the chlorothiazide concentration in the medium is increased, there is a decrease in the glucose disappearance rate from the medium (5). The following study delineates in greater detail additional changes in carbohydrate metabolism of dog aorta which are brought about by chlorothiazide, with special attention to glycogen and lactic acid.

Materials and methods. Mongrel dogs of both sexes weighing 18 to 27 kg were anesthetized with 20 to 30 mg of sodium pentobarbitol/kg body weight iv and exsanguinated, and the abdominal aorta was removed and placed in ice-cold isotonic saline solution. The aorta was cleaned of fat and adventitia, blotted, and 250- to 300-mg portions of tissue were placed in tared flasks previously gassed with 5% CO₂-95% O₂ mixture. Each flask contained 5 ml of Krebs' bicarbonate buffer solution (6) and was kept briefly in ice until ready for incubation at 37° for 2 hr.

In experiment I, the incubating medium also contained 11.1 mM glucose; in experiment II, there was no glucose in the medium, and in experiment III, the medium contained 1.2 mM lactic acid. In all experiments, one-half of the flasks contained 1.0 mM chlorothiazide in the medium. Glucose

determinations on medium before and after incubation were done by the glucose oxidase method (reagents from Worthington Biochemical Corp.). Lactic acid contents were done on the medium at the beginning and end of incubation by ultraviolet spectroscopy (Sigma Technical Bulletin No. 826). Glycogen content of tissue at the end of incubation was determined by its hydrolysis to glucose (7) which was then measured by the glucose oxidase method. Glycogen was calculated from glucose by the Morris conversion factor of 1.11 (8). Statistical significance was evaluated by Student's *t* test.

Results. In experiment I in which the medium contained glucose in eight studies, each composed of 5 to 10 control and chlorothiazide-containing flasks, mean glucose utilization of aorta was significantly reduced by the presence of 1 mM chlorothiazide in the medium (Table I). Mean net lactic acid production by aorta also was reduced significantly by the presence of 1 mM chlorothiazide in 11 studies, each composed of five to nine flasks (Table I). In five studies in which glycogen content of aorta was determined, the presence of 1 mM chlorothiazide in the medium resulted in a significantly lower mean glycogen content of the tissue at the end of incubation (Table I).

In experiment II in eight studies, each composed of four to eight control and chlorothiazide-containing flasks, mean net lactic acid production by aorta (in this system not containing glucose as substrate) was not reduced by the presence of 1 mM chlorothiazide (Table I). It should be noted that even in the absence of chlorothiazide the lack of glucose in the medium caused a considerable reduction in the net lactic acid production by aorta compared to that of aorta in medium containing 11.1 mM glucose ($P < 0.001$). In four studies in which glycogen content of aorta was determined at the end

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TABLE I. NET GLUCOSE UTILIZATION, LACTIC ACID PRODUCTION, AND GLYCOGEN CONTENT OF AORTA WITHOUT AND WITH CHLOROTHIAZIDE IN THE MEDIUM.^a

Experiment	Without chlorothiazide			With chlorothiazide ^b		
	Glucose (μ mole/g aorta/2 hr)	Lactic acid (μ mole/g aorta/2 hr)	Glycogen (μ g/g aorta)	Glucose (μ mole/g aorta/2 hr)	Lactic acid (μ mole/g aorta/2 hr)	Glycogen (μ g/g aorta)
I. Glucose in medium	30.0 \pm 0.81 (54)	46.6 \pm 0.84 (67)	878 \pm 64 (30)	23.3* \pm 0.84 (52)	42.7** \pm 0.83 (71)	603** \pm 55 (32)
II. No glucose in medium		24.7 \pm 0.54 (46)	150 \pm 6.1 (24)		25.5 \pm 0.63 (45)	156 \pm 10 (26)
III. Lactic acid in medium		26.7 \pm 0.63 (39)	177 \pm 16 (27)		24.2** \pm 0.66 (37)	184 \pm 21 (27)

^a Values expressed as Mean \pm SE. Number of flasks is in parentheses.^b Significantly different from medium without chlorothiazide: * $P < 0.001$; ** $P < 0.01$.

of incubation in medium not containing glucose, the presence of chlorothiazide resulted in no consistent change (Table I). As was the case with lactic acid production, in medium not containing chlorothiazide, the absence of glucose resulted in the glycogen content of the aorta at the end of incubation being greatly reduced ($P < 0.001$), as compared to that found with aorta incubated in glucose-containing medium.

In experiment III in which lactic acid was added to the medium in six studies, each composed of five to nine control and chlorothiazide-containing flasks, mean net lactic acid production by aorta was significantly lower in the presence of chlorothiazide (Table I). Note that all of these values represent an increase in lactic acid content of the medium at the end of incubation. In five studies in which glycogen content of aorta was determined at the end of incubation in medium containing 1.2 mM lactic acid, the presence of chlorothiazide resulted in no significant difference (Table I).

Discussion. Chlorothiazide interferes with the utilization of glucose by aorta as evidenced by the decreased rate of disappearance of glucose from the medium. When chlorothiazide is present in medium containing glucose, at the end of incubation there is both a decreased concentration of lactic acid in the medium and less glycogen present in the tissue. However, when no glucose is in present in the medium, so that final glycogen content of aorta is reduced and lactic acid production is decreased, then

the presence of chlorothiazide does not significantly alter either tissue glycogen content or net lactic acid production of aorta. The major action of chlorothiazide appears to be interference with the rate of utilization of glucose by the cells. This could be due to a slowing of the entry of glucose into the cells or a partial block of one or more metabolic steps within the cell.

It is known that the administration of thiazides to patients causes a reduction in circulating insulin-like activity (9). The *in vitro* experiments presented here, along with previous studies of fat tissue (3), indicate that in addition to having an effect on insulin-like activity chlorothiazide also acts at the cellular level independently of the insulin effect to decrease the rate of utilization of glucose. Furosemide has been shown to have similar *in vivo* and *in vitro* effects on glucose metabolism (10). Recently Jung and Mookerjee (11) have demonstrated that furosemide, and to a lesser degree chlorothiazide, interferes with the transport of glucose into red blood cells. This may well be the case with aorta and could explain the findings in this study. However, interference with enzymatic activities in the metabolic pathway of glucose can also play a role.

In cell-free systems, furosemide inhibits lactate formation from fructose-1,6-diphosphate and glucose-6-phosphate (12). This inhibitory action of furosemide appears to involve glyceraldehyde-3-phosphate dehydrogenase (12, 13). It does not seem likely, however, that the alterations in carbohy-

drate metabolism of aorta brought about by chlorothiazide are due primarily to inhibition of an enzymatic process. A better possibility would appear to be that chlorothiazide has a major effect on the entry of glucose into the cells.

Summary. Chlorothiazide in the incubating medium significantly reduces net glucose utilization, glycogen content, and net lactate production of normal dog aorta. When no glucose is present in the incubating medium, these actions of chlorothiazide are not evident. When 1.2 mM lactic acid is present in place of glucose as substrate, net lactic acid production of aorta is decreased by chlorothiazide and glycogen content is unaltered.

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Affinity of Human Fibroblast Interferon for Blue Dextran (39574)

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Recently, several investigators have reported the ability of interferon to bind to various substances (1-3) and have indicated that such methods are of value in the purification process. Furthermore, information relative to such interactions is useful in defining the characteristics of the interferon molecule. When estimating the molecular weight of crude interferon, we first realized that human fibroblast interferon binds to blue dextran (BD), the marker used to estimate void volume in gel chromatography. (BD is a sulfonated polyaromatic dye bound to dextran, a cross-linked complex of dextrose polymers). We have studied the affinity of BD for human interferon and find that the binding can be reversed through the addition to KCl. In this communication, we will report our studies on the interaction between blue dextran and human interferon.

Methods. Blue Dextran Crystalline, Pharmacia Chemicals (Piscataway, N. J.), was dissolved in distilled water at a concentration of 1 mg/ml, passed through a 300-nm Millipore filter, and stored at 4° in 50-ml aliquots.

Interferon preparations. Interferon was induced with a complex of polyriboinosinic acid-polyribocytidylic acid (IC) and diethylaminoethyl dextran (DEAE-D) in human muscle skin fibroblasts (MSF) subsequently maintained in Liebovitz L-15 media supplemented with 5% fetal calf serum (FCS) and glutamine (30 µg/ml), arginine (90 µg/ml), glucose (1 mg/ml), potassium penicillin G (150 U/ml), and streptomycin sulfate (250 µg/ml). This method has already been reported in detail (4, 5).

Interferon assay. Interferon was assayed by the microtiter technique employing MSF cells and vesicular stomatitis virus as already described (4).

Gel chromatography. Molecular weights were determined on Sepharose 6B and

Sephadex G-200 superfine gels in 1.6 by 20-cm columns calibrated with known molecular weight markers. The eluant was 0.05 M phosphate-buffered saline (PBS); the fraction volume, 1 ml; and the running temperature, 6°. At the conclusion of the experiment, each fraction was assayed for interferon.

Differential ultracentrifugation. Five-milliliter quantities of blue dextran solution were centrifuged at 50,000 rpm for 4 hr in the Beckman L2-65B ultracentrifuge with a SW65 rotor. The supernatant was removed and the pellet was suspended in 0.5 ml of distilled water. The process of Millipore filtration and preliminary centrifugation necessary to sterilize the BD and to eliminate smaller molecules was found to eliminate 50% of the BD originally present. Then 1 ml of an interferon preparation, either at pH 7.0 or acidified to pH 2.8 with citric acid, was added and the mixture was allowed to incubate overnight. The following day, sufficient quantities of either PBS, KCl, nicotinamide adenine dinucleotide (NAD) or adenosine 5'-triphosphate (ATP) solutions, for interferon at pH 7.0, or 0.1 M citric acid, for acidified interferon, were added to fill the respective ultracentrifuge tubes. After agitation to achieve even distribution of the contents, centrifugation was performed for a further 4 hr at 50,000 rpm. At the completion of this procedure, the upper 4.5 ml of supernatant was harvested and the pellet was resuspended in the lowest 0.5 ml of the solution. Pellet and supernatant either were dialyzed against PBS overnight (citric acid or KCl solutions) and then assayed for interferon activity, or were assayed directly (PBS solutions). To calculate the amount of interferon in the unsuspended pellet, the quantity of interferon in 0.5 ml of supernatant was subtracted from the total activity of the pellet solution.

Protein determinations. Protein concen-

trations were determined by the Lowry method (6).

Results. It can be seen in Fig. 1 that chromatography of interferon on Sephadex G-200 in the presence of blue dextran resulted in most of the recoverable bioactivity appearing in the void volume along with the BD. Similar results occurred on Sepharose

6B gels indicating a molecular weight of greater than 4×10^6 for both interferon and BD. In contrast, in the absence of blue dextran the recoverable interferon bioactivity peaked beyond the void volume on both gels and yielded an estimated molecular weight for MSF-IC interferon of 300,000.

Upon ultracentrifugation of MSF-IC interferon for 4 hr at 50,000 rpm, the interferon bioactivity remained in the supernatant (Table I). On the other hand, when the sedimentation was carried out in the presence of blue dextran (0.5 mg/ml), all of the BD and most of the interferon bioactivity appeared in the pellet (Table I). Thus, it appears that interferon binds to BD under the given conditions. Furthermore, with the quantity of BD and the volume constant, the amount of interferon bound varied linearly with the amount of interferon added. Somewhat similar results occurred at pH 2.8 with previously acidified interferon (Table I), indicating that the low molecular weight form of MSF-IC we have demonstrated at acid pH also binds to BD.

An attempt was made to free interferon bound to BD by increasing the ionic strength of the solution. The results when interferon bound to BD was exposed to

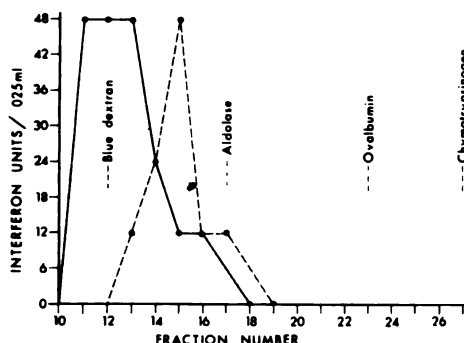


FIG. 1. Chromatography of MSF-IC on G-200 superfine gel at 6° with and without blue dextran. Column is calibrated with known molecular weight markers as indicated. One-half of the sample is applied to the column and counting is begun. Fraction size is 1 ml. Repeat testing gave similar results. (—) With blue dextran, 20,000 units applied, 30% recovery, (---) without blue dextran, 30,000 units applied, 15% recovery.

TABLE I. DISTRIBUTION OF INTERFERON ACTIVITY FOLLOWING ULTRACENTRIFUGATION WITH AND WITHOUT BLUE DEXTRAN.

		Interferon units added	Interferon units recovered		units bound mg blue dextran ^a
Interferon diluent			Pellet	Supernatant	
A. Blue dextran absent					
pH 7.0	0.15 M PBS	2,400	120	2,400	—
	0.15 M PBS	19,200	60	10,080	—
	0.15 M PBS	42,240	60	42,240	—
	0.15 M PBS	80,000	60	80,000	—
B. Blue dextran ^b present					
pH 7.0	0.15 M PBS	2,400	1,920	600	768
	0.15 M PBS	3,840	3,840	600	1,536
	0.15 M PBS	9,600	9,540	660	3,816
	0.15 M PBS	19,200	19,800	1,320	7,920
	0.15 M PBS	42,240	30,480	2,640	12,192
	0.15 M PBS	76,800	75,600	1,320	30,240
	0.01 M NAD ^c	76,800	75,600	1,320	30,240
	0.01 M ATP ^c	76,800	76,200	660	30,480
	L-15 Media	9,600	7,440	2,640	2,976
	pH 2.8	0.1 M Citric acid	9,600	3,600	2,640

^a Calculated using 2.5 mg of BD, the amount remaining after preliminary centrifugation.

^b For samples diluted in PBS, the difference in the proportion of interferon found in the pellet is highly significant when BD is present compared to when it is absent. $P < 0.01$ by Student's t test.

^c In 0.15 M PBS.

varying concentrations of KCl immediately prior to and during sedimentation are recorded in Table II. It can be seen that interferon was maximally released at 2 M concentrations. In contrast, interferon was not released by solutions of ATP or NAD.

It was found that the attachment of interferon to blue dextran with subsequent release in 3 M KCl results in 3.5-fold purification (Table III).

Discussion. The present work describes the binding and release of human IC-induced fibroblast interferon to a sulfonated polyaromatic dye. The ability of interferon to bind to blue dextran at pH 7 is clearly

shown both by gel chromatography and by ultracentrifugation. In both cases the addition of blue dextran changes the location of the interferon so that antiviral activity is associated with the blue dye either in the void volume or the pellet, respectively. Crude MSF-IC interferon probably binds to other proteins at pH 7.0 to give the large molecular weight form. We have previously shown that at pH 2.8, MSF-IC interferon has a molecular weight of 14,000 and therefore is presumed to have dissociated from other proteins (7). It is assumed that both the large and small molecular weight forms bind to BD, since even at this lower pH the majority (58%) of the interferon activity recovered is found associated with the blue dextran. Additionally, we have found that MSF-IC interferon partially purified by gel chromatography also attaches to blue dextran. We have not attempted to investigate the kinetics of the binding reaction rigorously but have detected similar binding when the incubation of BD and interferon prior to molecular separation has been omitted.

The binding reaction is clearly dependent on the ionic strength of the solution. By centrifugation of the interferon in the presence of blue dextran and treatment of the

TABLE II. THE RELATIONSHIP OF KCl CONCENTRATION TO THE RELEASE OF MSF-IC INTERFERON FROM BLUE DEXTRAN.

KCl conc (molarity)	Total recoverable interferon*	
	% Attached	% Released
0.01	100	0
0.01	100	0
0.50	63	37
1.00	27	73
2.00	8	92
2.50	8	92
3.00	8	92

* The interferon concentration used in these experiments varied from $10^{4.6}$ to $10^{5.2}$ units/ml.

TABLE III. PARTIAL PURIFICATION OF INTERFERON BY ATTACHMENT TO BLUE DEXTRAN AND ELUTION WITH 3 M KCl.

	Interferon		Protein total (mg)	Specific activity (units/mg)	Purification	% Recovered
	Total (units)	Concn (units/ml)				
Experiment A						
A. Crude interferon	9600	9600	4.2	2286	1.0 ×	—
B. Centrifugation of interferon plus BD						
Supernatant	2400	480	2.6	923	0.4 ×	25%
Pellet*	NT	—	NT	—	—	—
C. Centrifugation of pellet resuspended in 3 M KCl						
Supernatant	4800	960	0.6	8000	3.5 ×	50%
Pellet	960	1920	NT	—	—	10%
Experiment B						
A. Crude Interferon	9600	9600	5	1920	1 ×	—
B. Centrifugation of interferon plus BD						
Supernatant	<1200	—	4	—	—	—
Pellet	9600	19200	NT	—	—	100%
C. Centrifugation of pellet resuspended in KCl						
Supernatant	4800	960	0.6	8000	4.2 ×	50%
Pellet	<800	—	NT	—	—	—

* The supernatant was totally removed from this pellet.

with 3 *M* KCl, a 3.5-fold increase in ic activity can be attained.

Thompson *et al.* (8) have recently suggested that binding of proteins to blue dextran may be either to a specific dinucleotide contained within the molecule or via an ionic mechanism. Proteins containing the leucine fold should be eluted from blue dextran after exposure to low concentrations (0.01 *M*) of nucleotide ligands (8) such as ATP and NAD. It is clear from our results that the NAD and ATP solutions do release interferon from blue dextran, suggesting that the binding is ionic in nature.

Summary. Human fibroblast interferon has been shown to bind to blue dextran with a binding that is reversible on the addition of KCl. Attachment of human fibroblast interferon to blue dextran and subsequent elution results in a 3.5-fold purification with recovery.

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Effect of Pinealectomy and of Bilateral Cervical Ganglionectomy on Serum LH Levels in Constant Estrous-Anovulatory Rats (39575)

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It has been previously reported that pinealectomy and superior cervical ganglionectomy are able to reinstitute ovulation in constant estrous-anovulatory (CEA) rats bearing a frontal hypothalamic deafferentation (FHD) (1, 2). Repeated ovulations and pregnancies have been shown to occur in these animals (1, 2). However, the interval between two consecutive ovulations is not constant and the vaginal cycle is completely irregular (1, 2).

FHD has been reported to block the release but not the synthesis of pituitary LH (3). On the contrary, in normal rats pinealectomy has been shown to cause a marked rise in the synthesis as well as in the release of LH; the administration of the pineal principle melatonin exerts the opposite effects (4-8).

The present experiments have been designed to test whether pinealectomy and superior cervical ganglionectomy reinstitute ovulation in CEA rats bearing a FHD by activating the release of LH. In order to verify this hypothesis, serum levels of LH have been measured at different hours of the day in animals made CEA by FHD and subsequently submitted either to pinealectomy or to superior cervical ganglionectomy.

Materials and Methods. Female virgin rats of the Wistar strain were used. The animals were maintained throughout the experiment on a 14:10 hr light-dark cycle (lights on

from 6 AM to 8 PM), in a temperature and humidity controlled room ($24 \pm 1^\circ$); they were fed a standard pellet diet; water was allowed *ad libitum*. At the age of 10 weeks, the animals were submitted to a frontal deafferentation (FHD) behind the optic chiasm using the technique of Halász and Pupp (9). The characteristics of the knife were as follows: rotational radius of the blade, 1.5 mm, height of the blade, 2.0 mm. The animals were allowed approximately 4 weeks to establish the CEA syndrome. After that interval, vaginal smears were recorded daily. Only CEA rats which showed persistent vaginal cornification for at least 1 month (about 50% of the rats subjected to FHD) were submitted to further surgical procedures. These CEA rats were divided randomly into four groups and were subjected to pinealectomy (30 rats), or to bilateral superior cervical ganglionectomy (30 rats), or to the corresponding sham-operations (15 rats). The surgical procedures were performed under barbital anesthesia (Evipan Natrium Bayer, Leverkusen), according to the techniques previously described (1, 2). As a consequence of anesthesia and of the operations, the vaginal smears of CEA animals acquired the characteristics of diestrus for a variable number of days (from 2 to 10). Subsequently, the vaginal smears of pinealectomized and of ganglionectomized rats showed the appearance of an irregular cyclicity. These phenomena have been previously reported (1, 2). On the day in which the vaginal smears of these animals showed for the second time the characteristics typical of proestrus (this day was named of "vaginal proestrus"), blood samples were collected four times to test whether the release of ovulatory amounts of LH had occurred. Blood collections were performed by puncture of the retroorbital plexus, under light ether anesthesia at 10

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at 2, 4, and 6 PM. These times were used for sampling because, under the conditions adopted and in the strain used, the ovulatory surge of LH normally occurs between 2 and 4 PM. Ovulation was checked on the next morning by investigating the presence of ova in the oviducts. In accordance with previous findings (1), ovulation occurred the number of ova was within the range found in all animals of the same strain. The location and the completeness of the fronto-ventral afferentation were carefully checked by histological verification.

LH was measured in the serum using the γ -antibody radioimmunoassay procedure described by Niswender *et al.* (10). An antiserum against ovine LH, and pure ovine LH labeled with ^{125}I were used. Values have been expressed in the tables in terms of NIH-S 17 (conversion factor to NIH-1 = 1.01). The lowest amount of LH detectable with the procedure here adopted was of the order of 0.1 ng/ml.

Results. As shown in Table I, pinealectomy was able to induce ovulation in 65.5% of CEA rats; almost the same figure was obtained in CEA rats subjected to bilateral resection of the superior cervical ganglia. Table II summarizes the values of serum LH found in pinealectomized FHD rats that had ova in the oviducts. For comparison, serum LH levels of normal control animals have also been included in the table. Of 19 pinealectomized animals, 9 showed some sort of fluctuation in their serum LH levels. Three animals (Nos. 1, 5, and 11) presented LH peaks around the time at which these appear in normal animals of the strain used in the present series of experiments (2–4 PM). In three other animals (Nos. 6, 10, and 15), LH concentrations reached a maximum at 6 PM. Three

other rats (Nos. 7, 9, and 17) showed the highest levels of serum LH at 10 AM. In the remaining 10 animals (Nos. 2, 3, 4, 8, 12, 13, 14, 16, 18, and 19), the concentrations of LH remained constant during the period in which blood was sampled on the day of "vaginal proestrus," in spite of the presence of ova in the oviducts the following day.

The concentrations of LH in the sera of FHD rats, which ovulated after ganglionectomy, are reported in Table III. Serum LH levels of normal controls are given also for comparison. Out of 16 ganglionectomized animals, 7 showed the presence of elevated levels of LH. Two animals (Nos. 6 and 8) showed the elevation around the time at which the LH surge occurs in normal animals. Two other rats (Nos. 1 and 7) exhibited the highest levels of serum LH at 10 AM. Finally, three other animals (Nos. 3, 10, and 14) had an irregular behavior of their serum LH titers with a tendency to reach the highest values in the late afternoon. The remaining rats (Nos. 2, 4, 5, 9, 11, 12, 13, 15, and 16) did not show significant variations of serum LH values.

Table IV summarizes the data obtained in animals bearing a FHD and subsequently submitted to pinealectomy and to superior cervical ganglionectomy and which did not ovulate. It is interesting that not one of the animals belonging to these groups showed increased release of LH during the day of "vaginal proestrus." Consequently, we have pooled these results. It is also interesting to note that in these two groups of animals (and especially in those submitted to ganglionectomy), serum LH levels were lower than in FHD-sham-operated animals. Since plasma LH levels in CEA rats do not change throughout the day, only a single blood collection was made in the last group of animals.

Discussion. The observation that 65% of the CEA rats ovulated following either pinealectomy or superior cervical ganglionectomy confirms previous reports (1, 2, 11). While 50% of the animals which ovulated following either operation showed elevated serum LH levels during the day of "vaginal proestrus," the time at which this elevation occurred was not identical in all animals. In one-third of these, LH increased at the time LH surges occur in normally cycling rats (2–

TABLE I. OVULATIONS INDUCED BY PINEALECTOMY AND SUPERIOR CERVICAL GANGLIONECTOMY IN CONSTANT ESTROUS-OVULATORY (CEA) RATS BEARING A FRONTAL HYPOTHALAMIC DEAFFERENTATION (FHD).

Treatment	Ovulating rats/operated rats	%
pinealectomy	19/29	65.5
ganglionectomy	16/25	64
sham operations	0/15	0

TABLE II. SERUM LH LEVELS (NANOGRAMS PER MILLILITER) OF OVULATING PINEALECTOMIZED FHD RATS AT DIFFERENT HOURS OF THE DAY OF "VAGINAL PROESTRUS."

	10:00 AM	2:00 PM	4:00 PM	6:00 PM
Pinelectomized-FHD rats				
Rat no. 1	8.50	14.00	14.50	9.00
2	4.25	5.25	5.50	7.25
3	4.75	2.80	3.00	4.80
4	3.31	0.84	1.07	0.95
5	0.18	11.80	1.00	0.47
6	1.04	1.12	6.48	11.13
7	10.00	2.00	0.75	2.25
8	1.46	1.24	1.18	0.63
9	8.00	8.50	2.25	5.00
10	0.73	0.19	10.69	47.10
11	4.00	17.75	15.00	13.00
12	3.00	3.00	3.20	2.00
13	0.13	1.06	0.14	0.32
14	0.28	0.37	0.63	0.74
15	0.35	0.47	0.32	36.68
16	4.63	0.66	1.03	3.51
17	8.50	6.00	0.75	0.50
18	2.20	1.24	1.18	0.63
19	0.64	1.06	0.59	1.63
Normal controls ^a	13.30 ± 1.60	28.70 ± 10.00	35.05 ± 6.78	17.75 ± 2.69

^a Means ± SE of six animals.

TABLE III. SERUM LH LEVELS (NANOGRAMS PER MILLILITER) OF OVULATING GANGLIONECTOMIZED FHD RATS AT DIFFERENT HOURS OF THE DAY OF "VAGINAL PROESTRUS."

	10:00 AM	2:00 PM	4:00 PM	6:00 PM
Ganglionectomized-FHD rats				
Rat no. 1	13.50	7.75	3.25	5.50
2	3.82	1.07	1.11	1.28
3	0.51	8.22	12.24	38.87
4	2.50	2.75	2.25	3.00
5	0.88	1.03	1.08	1.12
6	3.73	3.09	32.89	23.73
7	13.00	11.00	2.75	0.50
8	8.50	35.50	50.00	21.00
9	3.82	1.98	1.12	0.91
10	0.45	6.42	1.26	10.42
11	0.32	0.88	0.71	0.64
12	1.46	0.21	0.33	0.64
13	2.50	3.50	4.00	5.00
14	7.22	3.00	6.08	10.51
15	1.00	3.25	1.00	4.75
16	0.75	1.20	1.00	1.75
Normal controls ^a	13.30 ± 1.60	28.70 ± 10.00	35.05 ± 6.78	17.75 ± 2.69

^a Means ± SE of six animals.

4 PM). In the second third, LH did not increase until 6 PM, whereas in the final third, serum LH was elevated at 10 AM. The remaining 50% of FHD rats which ovulated after pinealectomy or after superior cervical ganglionectomy did not exhibit any significant variation in serum LH during the hours of the day in which serum was collected. In FHD animals submitted to pinealectomy or to superior cervical ganglionectomy which

did not ovulate, serum LH levels remained unchanged.

In the majority of FHD rats which ovulated after pinealectomy or after superior cervical ganglionectomy and which exhibited an elevation of serum LH titers, this elevation did not approach the levels found in normally cycling animals. The only exception were rats Nos. 10 and 15 of Table II and rats Nos. 3, 6, and 8 of Table III. Three

TABLE IV. SERUM LH LEVELS (NANOGRAMS PER MILLILITER) OF NONOVULATING PINEALECTOMIZED AND GANGLIONECTOMIZED FHD RATS.

Treatment	No. of rats	10:00 AM	2:00 PM	4:00 PM	6:00 PM
pinelectomy	10	3.50 ± 0.74 ^a	3.26 ± 0.55	3.75 ± 0.69	4.73 ± 0.87
ganglionectomy	9	1.86 ± 0.48 ^b	2.25 ± 0.52 ^c	2.25 ± 0.55 ^c	3.16 ± 0.82
sham operation	15		4.36 ± 0.47		

ns ± SE.

0.005 vs FHD sham-operated animals.

0.01 vs FHD sham-operated animals.

ulations may be offered to explain results. (A) It is possible that the modulation of LH found in the majority of pinealectomized and ganglionectomized rats is the consequence of the lower basal levels of LH observed by us and Hashi *et al.* (12). (B) The LH release occurs after pinealectomy or ganglionectomy is anomalous in nature, and it is due to a stimulus originating in the CEA area. Considerable evidence has been presented which suggests that FHD about the CEA syndrome by disconnecting the preoptic input into the arcuate nucleus region (13). Pinealectomy induces ovulation through a depression of activity of the serotonergic system in the brain stem (14–16), which normally inhibits the release of LH (7, 17–20). In support of such a hypothesis, the following may be quoted: injections of serotonin and melatonin are able to suppress the LH-releasing effect of pinealectomy in rats (11, 14); the administration of serotonin increases the concentrations of LH in the hypothalamus and in the blood (21); the depression of serotonin levels induced by treatment with parachloroamphetamine or by feeding with a choline-poor diet brings about luteinization in CEA-FHD rats (15, 16). Finally, (C) it is possible that in some animals (particularly those in which LH levels were high at 10:00 AM or at 6 PM) the peak LH values were because of inadequate frequency of sampling of blood. Consequently, in these rats only the ascending or the descending phases of the LH surge, respectively, were measured.

Our data also indicate that small elevations of serum LH are sufficient to induce

ovulation in CEA rats bearing a FHD. This is not surprising since it is well known that the serum LH concentrations which occur in normal rats on the afternoon of the day of proestrus far exceed the threshold amounts needed to induce ovulation (22–25).

Summary. In 65% of constant estrous non-ovulatory (CEA) rats bearing a frontal hypothalamic deafferentation (FHD), ovulation reappears after pinealectomy or bilateral superior cervical ganglionectomy. In nearly 50% of the FHD rats which ovulated after pinealectomy or ganglionectomy, serum LH showed an elevation on the day of "vaginal proestrus." In the remaining 50% of the animals, ovulation occurred without observable variations in serum LH concentrations. The LH peak was probably missed in these animals because insufficient numbers of blood samples were collected. The data suggest that pinealectomy and cervical sympathectomy may induce LH release. It is proposed that this occurs through an interference with the hypothalamic and brain stem serotonergic system which normally keeps LH under an inhibitory tone.

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Cytotoxicity of Lymphocytes in Experimental Alcoholic Liver Injury in the Baboon¹ (39576)

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Although immunologic reactions and a cytotoxic activity of lymphocytes against the animal's own liver cells have been considered an important factor in some chronic liver diseases (e.g., chronic active hepatitis, primary biliary cirrhosis) (1-5), little is known whether they play any role in the initiation and perpetuation of alcoholic liver disease. An altered cell mediated immunity against foreign antigens (6, 7) and a pronounced decrease in peripheral blood T lymphocyte numbers in patients with alcoholic hepatitis (8) have suggested a basic impairment in cell-mediated immunity in alcoholic liver diseases. These findings have called for further investigations directed at determining the role of immunological factors in the development and progression of alcoholic liver diseases.

The experimental model of baboons undergoing prolonged feeding with alcohol and covering the entire spectrum of alcoholic liver disease (9) provides a valuable system to test the hypothesis of the importance of immunological reaction in the development of alcoholic liver diseases.

Materials and Methods. Twenty-four baboons (12 alcohol treated and 12 pair-fed controls) were studied. Alcohol was administered as previously described (10) for a period ranging from 16 to 38 months. Lymphocyte-mediated cytotoxicity was studied as the *in vitro* correlate of cellular immunity. The cytotoxic activity of lymphocytes was investigated against liver cells derived from the animal's own liver, thus eliminating a reaction due to histocompatibility differences. Lymphocytes were isolated through a Ficoll-Hypaque gradient (11), and incubated for 16 hr in plastic con-

tainers at 37° in 5% CO₂ saturated with water vapor and further purified through nylon column (12). Cytotoxicity was measured first by the microcytotoxic test of Takasugi and Klein (13) based on the destruction or detachment of adherent target cells by purified lymphocytes and evaluated by counting the target cells that remain attached. In later experiments, the method of Bean *et al.* (14) was employed. This method measures cytotoxicity by counting residual [³H]proline label in cultured liver cells after 2 days of incubation with purified lymphocytes. After liver biopsy, liver cells were cultured according to the method of Demoise *et al.* (15).

Results. Liver cells from all 12 control pair-fed animals formed a monolayer within 4-5 weeks and could be easily trypsinized and subcultured. Liver cells from alcohol-treated animals were much more difficult to establish; only 6 of 12 cell lines could be subcultured. Histologic examination of these livers revealed marked steatosis and portal fibrosis. One liver exhibited moderate accumulation of lymphocytes in the portal tract and in the parenchyma. No alcoholic hepatitis or cirrhosis was observed in these animals. The cells of three animals with incomplete cirrhosis and/or hepatitis did not grow. Lymphocytes of alcohol-fed baboons exhibited cytotoxicity against autochthonous liver cells, as detected by the morphologic (Table I), and the radioisotopic (Table II) methods. Cytotoxicity was observed only if a lymphocyte to liver cell ratio of 100:1 was utilized. When the number of lymphocytes was decreased, no cytotoxicity was observed (Table I). Lymphocytes of control pair-fed animals did not display cytotoxicity.

Discussion. Lymphocytes of alcohol-treated baboons acquire cytotoxicity against

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TABLE I. CYTOTOXICITY OF BABOON LYMPHOCYTES AGAINST AUTOCHTHONOUS LIVER CELLS GROWN IN TISSUE CULTURE.^a

Treatment	Length of treatment (months)	Lymphocyte cytotoxicity	
		L:T ^b = 10:1	L:T ^b = 100:1
Alcohol	16	12	70 ^c
Alcohol	20	11	48 ^c
Control		7	5
Control		10	7

^a Monolayer was incubated for 2 days with lymphocytes. Cytotoxicity measured as percentage reduction of number of hepatocytes incubated with lymphocytes in relation to control hepatocytes (2).

^b Lymphocyte to target cell ratio.

^c $P < 0.001$.

TABLE II. CYTOTOXICITY OF BABOON LYMPHOCYTES AGAINST AUTOCHTHONOUS LIVER CELLS GROWN IN TISSUE CULTURE

Treatment	Length of treatment (months)	Lymphocyte cytotoxicity ^a
Alcohol	21	1.16 ^b
Alcohol	34	2.20 ^b
Alcohol	32	1.10 ^b
Alcohol	38	1.40 ^b
Control		0.97
Control		0.98
Control		1.00
Control		1.04

^a Monolayers of [³H]proline-labeled hepatocytes were incubated for 2 days with lymphocytes. (Ratio of lymphocyte to target cells, 100:1.) Cytotoxicity is expressed as counts per minute (cpm) control hepatocytes/cpm hepatocytes with lymphocytes. Method according to Bean *et al.* (14)

^b Changes statistically significant: $P < 0.001$.

autochthonous liver cells before alcoholic hepatitis and cirrhosis ensues. It appears important in these experiments to utilize cells derived from the animal's own liver.

Pilot experiments attempting to use allogeneic liver cells revealed that lymphocytes of both control and alcoholic animals are cytotoxic. Most likely lymphocytes recognize histocompatibility differences still present in the cultured cells. In the present investigation, no attempts were made to establish the organ specificity of the cytotoxic reaction, why liver cells of alcoholic animals are established with difficulty, and whether they are more susceptible to injury.

Little is known about the acute or chronic effect of alcohol on lymphocyte activity. It

has been claimed that alcohol induces lymphocyte stimulation in patients with alcoholic hepatitis and chronic active hepatitis (6) and blocks lymphocyte stimulation by phytohemagglutinin (16). The present demonstration of cytotoxicity of lymphocytes against autochthonous liver cells before the development of alcoholic hepatitis or cirrhosis may have an important pathogenetic significance and warrants further investigations on the role of altered lymphocytic reactivity as a mechanism for the development of alcoholic liver disease.

Summary. To study the role of immunologic reactions in the development of alcoholic liver disease, cytotoxic activity of lymphocytes against autologous liver grown in tissue culture was investigated. The experimental model was that of ethanol-fed baboons which develop the entire spectrum of alcoholic liver disease. Liver tissue was obtained at laparotomy in 12 animals given alcohol for 16–38 months and in 12 pair-fed controls. Lymphocytes of all alcohol-treated animals (but not of the controls) exhibited cytotoxicity against autochthonous liver cells: there was a marked decrease in the number of surviving cells and a significant reduction in their [³H]proline label.

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Canine Pituitary Prolactin: Isolation and Partial Characterization (39577)

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For many years the only readily available prolactin preparations have been of ovine or bovine origin (1). Consequently, the bulk of chemical and physiological studies has been performed with these preparations. In recent years, isolation procedures have been described for obtaining prolactin from several other species: rat (2), pig (3, 4), and the human (5). The complete amino acid sequences are now known for ovine (6), bovine (7), and porcine (8) prolactin, and in a preliminary communication, the sequence of rat prolactin was reported (9). The availability of the above-mentioned species of prolactin have materially advanced structure-function as well as physiological studies of prolactin action by allowing, for instance, the development of radioimmunoassays. There is still a need, however, for other species of prolactin. One of these is canine prolactin, insofar as the dog is an extensively employed experimental animal. I wish to report here a simple procedure for the isolation of highly purified prolactin from a limited number of dog pituitaries. In addition, some of the properties of the canine prolactin were determined.

Purification. In this study, 300 (approximately 13.8 g) frozen dog pituitaries obtained commercially from Pel-Freez Co., Little Rock, Ark., were employed. The presence of prolactin in various fractions was detected by a mixed heterologous radioimmunoassay (porcine prolactin tracer; anti-ovine prolactin serum) which cross-reacted with canine prolactin (10) and by disc electrophoresis at pH 8.3 in 7.5% polyacrylamide gels. The pituitaries were homogenized in a Waring Blendor with 600 ml of H₂O, adjusted to pH 9.5 with CaO, and stirred overnight at 4°. Following centrifugation, the extract was adjusted to 0.15 M (NH₄)₂SO₄ and the pH to 4.0 by addition of freshly prepared 0.2 M HPO₃. The precipitate which formed was dialyzed against wa-

ter, lyophilized (yielding 2.1 g), and extracted with pH 5.1-0.45 M (NH₄)₂SO₄ buffer. The residue was suspended in 50 ml of H₂O, dialyzed against water, and adjusted to pH 10.0 with N NaOH. Insoluble material was centrifuged off and cold (-20°) EtOH was added to a concentration of 50% (v/v). The resultant precipitate was centrifuged off, the supernatant fluid was adjusted to pH 5.0 with N HCl, and 4 vol of cold EtOH was added. The ethanolic precipitate was then extracted with 0.05 M NH₄HCO₃ and the extract was applied to a column of Sephadex G-100 in 0.05 M NH₄HCO₃ for final purification. The major symmetrical peak which eluted with a *Ve/Vo* of 2.2 was lyophilized and used for characterization studies. A yield of approximately 6 mg was obtained from the 13.8-g wet weight pituitaries.

Biological characterization. The purified canine prolactin was assayed several times in pigeons by the local crop-sac test (11). The results showed that the canine prolactin preparation had a dose-dependent response which was nonparallel (a flatter slope) when compared with ovine prolactin. Its potency relative to ovine prolactin (taken as 30 units/mg) was between 11-27 units/mg. Contamination with growth hormone was estimated to be 0.87% as measured by radioimmunoassay (12).

Amino acid and NH₂-terminal group analyses. The amino acid content of the canine prolactin was determined by the method of Spackman *et al.* (13), following hydrolysis in 5.7 N HCl at 105° for 20 hr in sealed evacuated tubes. The results are shown in Table I and compared to several other species of prolactin. Noteworthy is the general similarity between the prolactins, particularly with respect to the half-cystine content (six in each), and the high content of leucine, aspartic acid, and glutamic acid. The methionine content of canine prolactin, however, is

TABLE I. AMINO ACID COMPOSITION OF CANINE PROLACTIN COMPARED TO OVINE, PORCINE, AND MURINE PROLACTIN.

Amino acid	Canine ^a	Canine ^b	Ovine ^c	Porcine ^d	Murine ^e
Lysine	7.0	9	9	9	13
Histidine	5.9	7	8	9	6
Arginine	12.5	13	11	13	8
Aspartic	24.2	22	22	22	23
Threonine	6.1	6	9	5	6
Serine	15.0	15	15	16	12
Glutamic	26.5	24	22	24	28
Proline	9.1	8	11	7	12
Glucine	11.6	11	11	8	7
Alanine	13.3	13	9	10	10
Cystine (one-half)	6.0	N.D.	6	6	6
Valine	9.6	11	10	11	12
Methionine	3.3	1	7	4	2
Isoleucine	10.3	11	11	15	14
Leucine	25.2	22	23	26	23
Tyrosine	5.4	6	7	7	7
Phenylalanine	6.8	6	6	6	7
Tryptophan	1.6 ^f	N.D.	2	2	2

^a Average of five determinations.

^b Data of Knight *et al.* (17).

^c Structural data of Li *et al.* (6).

^d Structural data of Li *et al.* (8).

^e Structural data of Parlow and Shome (9).

^f Determined spectrophotometrically.

somewhat lower than that found in ovine prolactin (three to four in canine vs seven in ovine) as are several other residues (lysine, histidine, threonine). Tryptophan content was determined spectrophotometrically (14) and the results suggest the presence of two residues as are present in ovine, porcine, and rat prolactin. Estimates of the molecular weight of the canine prolactin from amino acid analyses and gel filtration behavior on Sephadex G-100 suggest a value of about 22,000 which is consistent with that of ovine prolactin (1). Amino terminal group analysis by the dansyl-Edman procedure (15, 16) showed leucine to be the major terminal amino acid with a trace of phenylalanine.

Recently, Knight *et al.* (17) reported on the preparation of canine prolactin by means of the technique of isotachopheresis. Their preparation had a similar biological potency to that reported here. Their amino acid analysis (Table I) was incomplete (no values for half-cystine and tryptophan), but in general was similar to that reported here except for a much lower content of methionine (one vs three).

Summary. Highly purified canine prolac-

tin has been prepared and partially characterized. The material is comparable in potency to ovine prolactin, and similar in amino acid composition as well.

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Inhibition of Interferon Action by Cytochalasin B, Colchicine, and Vinblastine (395578)

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We have previously postulated that a membrane-bound receptor system is responsible for the initial steps leading to the viral protection induced by interferon. Additional experiments using insoluble interferon (2) or ouabain (3) support this thesis. The role of the receptor system is substantiated by the observation that antibodies directed against interferon block establishment of the antiviral state in interferon-producing cells (4). The dose-response curves using either soluble or insoluble interferon are sigmoidal, thus suggesting a cooperative event in antiviral action. This cooperative event could occur at the receptor level as shown by studies using all these drugs (1, 5).

Cooperative activation could involve dual receptors or a number of them, and it is ordered whether the mobility of such receptors is important or not in interferon action. The microtubule and microfilament systems, among other properties (6), have been associated with the mobility of membrane-bound proteins (7). It was therefore of interest to study the effect on interferon action of cytochalasin B, colchicine, and vinblastine, which are known to disrupt these systems (8, 9).

Materials and methods. Mouse L929 cells were cultivated in 35-mm petri dishes (7 × 10⁵ cells per dish) in Eagle's minimum essential medium (MEM) containing 10% calf serum. Murine interferon was produced in L cells induced by Newcastle disease virus (NDV) following standard procedures. The concentrations used in these experiments contained 600 interferon reference units (sp act 0.5 × 10⁴ units/mg of protein). The cells were treated with interferon and simultaneously with cytochalasin B (Calbiochem), colchicine (Sigma), or vinblastine (Sigma), alone or in combination at a final concentration of 5 μg/ml. After 4

hr of incubation, the medium and drugs were removed and cells were challenged with encephalomyocarditis virus (EMC) at a multiplicity of infection (m.o.i.) = 1 for 1 hr. The viral yield was measured by plaque titration after 18 hr of incubation at 37°.

Effect of the drugs on cellular RNA and protein synthesis was estimated as follows: cells were labeled with ¹⁴C-amino acid (protein hydrolysate of *Chlorella vulgaris*, sp act 810 μCi/mg) at a concentration of 0.2 μCi/ml, and [³H]uridine (sp act 25 Ci/mmol) at a concentration of 1 μCi/ml (both purchased from C.E.A. Saclay, France), which were added to confluent monolayers of L cells. Cytochalasin B, colchicine, or vinblastine were added simultaneously at a concentration of 5 μg/ml. After 4 hr of incubation at 37°, cells were washed three times with cold PBS, then 0.3% SDS was added for 20 min at 37°. TCA (10%) was added for 1 hr at 0°, and after centrifugation, the precipitate was washed with 5% TCA and dissolved in ammonium. Both the acid-soluble and insoluble fractions were counted using Bray's scintillator fluid.

Results and discussion. As shown in Table I, when L cells were treated simultaneously with murine interferon and either cytochalasin B, colchicine, or vinblastine, all three drugs significantly decreased the antiviral properties of interferon. However, the association of cytochalasin B either with colchicine or vinblastine inhibited even to a greater extent the antiviral action of interferon. The association of vinblastine and colchicine was less efficient.

Since for interferon action the integrity of cell protein synthesis is requisite, it was of importance to find out whether or not such a modification of the cell metabolism could explain the observed results. Therefore in a parallel series of experiments, we explored the effect on RNA and protein synthesis of

these three drugs, used alone or in combination. As shown in Table II, during the 4-hr duration of the experiment at the concentration employed, they did not produce detectable changes in the incorporation of ^{14}C -labeled amino acid into the cell.

In further experiments, when cells were labeled with $[^3\text{H}]$ uridine in the presence of the different drugs, no significant change in $[^3\text{H}]$ uridine incorporation was observed either in the cellular pool or in the acid-insoluble fraction, except in the case of cytochalasin B. When this drug was added to the cells in the presence of labeled uridine for 4 hr, a 50% decrease in uridine incorporation into RNA was observed. Since a similar decrease was noted in the acid-soluble frac-

tion, this reflected in fact a diminution in the intracellular pool of $[^3\text{H}]$ uridine. When the cells were labeled for 5 min and then washed prior to the addition of the drug, uridine incorporation in the acid-insoluble compartment was similar to untreated cells (Fig. 1). Thus, cellular RNA synthesis is not really involved in the anti-interferon effect of cytochalasin B.

Likewise, the inhibition of glucose uptake by cytochalasin B is probably not the explanation of the decreased response of the cells to interferon. The absence of glucose in the medium during the experiment did not affect the establishment of the antiviral state (unpublished experiments).

Previous investigations on the mechanism

TABLE I. EFFECT OF CYTOCHALASIN B, COLCHICINE, AND VINBLASTINE ON INTERFERON ACTION.^a

	Experiment 1		Experiment 2		Experiment 3	
	IF	MEM	IF	MEM	IF	MEM
Control	2.7×10^6	6.2×10^6	10^6	2.3×10^6	5.6×10^6	8×10^6
Cytochalasin B	2.5×10^7	7×10^6	2×10^6	3×10^6	2×10^7	1×10^6
Colchicine	1.7×10^7	4.6×10^6	—	—	2.7×10^7	3.3×10^6
Vinblastine	—	—	4×10^6	2.6×10^6	3.5×10^7	9×10^6
Cytochalasin B + colchicine	6.3×10^7	5×10^6	—	—	3×10^7	7.6×10^6
Cytochalasin B + vinblastine	—	—	1.3×10^7	1.5×10^6	7.2×10^7	7×10^6
Colchicine + vinblastine	—	—	—	—	4.4×10^7	8×10^6

^a L cells were treated with interferon (IF) and simultaneously with cytochalasin B, colchicine, and vinblastine, alone or in combination. After 4 hr of treatment, interferon and drugs were eliminated and cells were infected with EMC. The viral yield is expressed in plaque-forming units per milliliter. The standard error of the titration measured after 10 independent estimations of the same suspension is ± 0.11 log for $P < 0.01$.

TABLE II. EFFECT OF CYTOCHALASIN B, COLCHICINE, AND VINBLASTINE ON CELLULAR RNA AND PROTEIN SYNTHESIS.^a

	$[^3\text{H}]$ Uridine		^{14}C -labeled amino acids	
	Acid soluble fraction (324,376) ^d	Acid-insoluble fraction (121,041) ^d	Acid-soluble fraction (14,498) ^d	Acid-insoluble fraction (6368) ^d
Cytochalasin B	$58.66^b \pm 10.38^c$	$62.8^b \pm 13.13$	101.2 ± 13.77	99.2 ± 2.82
Colchicine	97.6 ± 2.85	103.4 ± 12.33	98.4 ± 9.77	100.8 ± 8.72
Vinblastine	99.66 ± 13.65	101.33 ± 12.23	93.33 ± 4.60	98.33 ± 10.32
Cytochalasin B + colchicine	$53.5^b \pm 10.49$	$58^b \pm 13.46$	93.60 ± 4.42	103.4 ± 12.17
Cytochalasin B + vinblastine	$70.5^b \pm 6.42$	$71.5^b \pm 2.75$	92 ± 2.42	90.5 ± 8.25
Vinblastine + colchicine	96.66 ± 2.85	100.33 ± 2.48	95.33 ± 13.01	95.66 ± 11.93

^a ^{14}C -labeled amino acid and $[^3\text{H}]$ uridine incorporation in cellular RNA and protein was measured as described in Materials and Methods. The results are expressed in percentage as compared to the control and calculated on the basis of three independent experiments.

^b Statistically different from the control for $P < 0.01$.

^c Each entry represents mean \pm SE for $P < 0.05$.

^d cpm in control cells (mean of three experiments).

on of the three drugs here studied shown that they are able to modify response to lectins in the absence of detectable competition for binding in the case of both leukocytes (10) and normal cells (11), colchicine or vinorelbine decreased cell agglutination promoted by concanavaline A. Similar findings obtained with cytochalasin B (12). These results suggested that topographic modifications in the membrane-bound receptors induced by the drugs could explain their effect. In addition, using freeze-etch electron microscopy, a modification in the distribution of membrane-intercalating particles was observed after treatment with colchicine (13).

In our studies, although the drugs were used alone, their potency increased when associated. Blockage of interferon action was always obtained in the absence of detectable modification of cellular RNA or protein synthesis. This view is also supported by the observation that at concentrations previously employed, colchicine and vinblastine (4) and cytochalasin B (15) do not decrease interferon synthesis. Thus, the integrity of microfilaments and microtubules would be necessary for the induction of the antiviral effect by interferon. They could modify the stability of membrane-interferon receptors or prevent an interaction between receptors or other components requisite for the initiation and amplification of the cellular response to this virus. Likewise, the disruption of microtubules could interfere with receptor-cytosolic interactions as previously suggested (16-18).

It is unlikely, however, that the known modifications in the active uptake of glucose by cytochalasin B could explain our results. The observed decrease of uridine uptake is probably also not involved in the loss of antiviral effect of interferon. In our experimental conditions, no change in the acid uptake is noted in contrast to a previous report (19), perhaps because of the lower drug concentrations employed.

It can be concluded from our studies that the interferon-induced decrease of uridine uptake is due to modifications of the cell membrane structure. This could be related

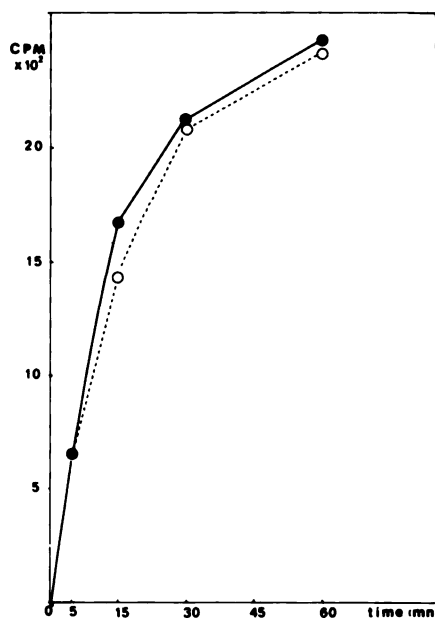


FIG. 1. Incorporation of [3 H]uridine into RNA in the presence of cytochalasin B. [3 H]Uridine (5 μ Ci/ml) was added to L cells for 5 min at 37°. After removal of uridine and three washings with MEM, cytochalasin B (5 μ g/ml) was added. At different time periods, cells were washed in cold PBS and treated as described in Methods. ●—● MEM; ○—○ cytochalasin B.

to the inhibitory effect of ouabain on the interferon-induced antiviral state (3). It is also possible, however, that other presently unknown properties of these inhibitors could contribute to explain these results.

Summary. Cytochalasin B, colchicine, and vinblastine decrease the antiviral effect of interferon when added to cells simultaneously with interferon. The drugs are effective alone but their potency increases when used in combination. The lack of development of the antiviral state is observed without any detectable modifications in cellular RNA or protein synthesis. These drugs are known to disrupt microtubules and microfilaments which are therefore probably necessary for interferon action. They could be involved in the distribution of membrane-associated receptors and/or in interactions between receptors and the cytoplasm. The initial steps leading to the establishment of antiviral protection by interferon could depend on these mechanisms.

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ated Erythrocytes of Cholesterol-Fed Guinea Pigs: Changes of Morphology, Composition, and Osmotic Fragility¹ (39579)

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ery cholesterol induces a hemolytic in guinea pigs (GP) preceded for weeks by fatty infiltration of the liver, changes in liver, plasma, and erythrocyte (RBC) cholesterol contents, and by changes in RBC morphology (1, 2). The relationship between the morphological abnormalities of the cholesterol-loaded RBC is not well understood, but changes in cell-shape have been reported to accompany increased plasma cholesterol both in human patients (3-5) and in *in vitro* systems (6, 7). The relationship would be strengthened if the course of shape change and alteration of cholesterol content could be shown to be similar. Maintenance of normal red blood cell shape has been shown to require normal levels of ATP and ATPase activity (8, 9). Therefore, if proper functioning of energy-producing reactions. Changes in membrane composition may be associated with changes in enzyme activities. This study was designed to compare sequential changes in RBC morphology with the cholesterol concentration in guinea pig RBC in response to dietary cholesterol. Concentration of ATP and activities of three key enzymes in the hemolytic sequence and of ATPases were measured simultaneously.

The mechanism for the development of hemolytic anemia in cholesterol-fed guinea pigs (GP) has not been established. Changes in fragility of cholesterol-loaded RBC might be one possible mechanism. Osmotic fragility has been shown to be altered in patients with obstructive jaundice and in experimental liver disease associated with an increase of RBC cholesterol (4, 10). We have measured changes in the osmotic fragility of guinea pig RBC in response to dietary cholesterol.

This work was supported in part by USPHS, NIH Grant 08480, and was presented in part at the 58th Meeting of FASEB, Atlantic City, April 1974.

Materials and methods. Male guinea pigs (200 g) were fed *ad libitum* Purina guinea pig chow supplemented with 5% cottonseed oil for 4 weeks prior to the addition of 1% cholesterol to the diet of the experimental group (11). Blood was collected either directly from the heart or from the ear vein using disodium ethylenediaminetetraacetic acid (EDTA) or heparin as anticoagulant, respectively.

Morphology of the RBC was assessed in whole blood. One drop of blood was diluted into 1.0 ml of Hayem's solution (9 mM HgCl₂, 75 mM Na₂SO₄, 85 mM NaCl). Slides of fixed cells were photographed using phase contrast optics and RBCs were classified into categories as summarized in Fig. 1.

Cholesterol of plasma and RBC, washed twice with isotonic saline, was extracted, precipitated as the digitonide, and assayed using the ferric chloride colorimetric method (12).

Osmotic fragility of red cells was determined in freshly drawn whole blood. Blood was added to tubes containing varying concentrations of NaCl (0-0.75%, w/v). The absorbance of the supernatant solutions was read at 540 nm after 60 min.

ATP content of whole blood, and the activities of hexokinase (Hex), phosphofructokinase (PFK), pyruvate kinase (PK), and of Na⁺-, K⁺-, and Mg²⁺-stimulated ATPases were measured by published methods (13, 14).

Results. Morphology and cholesterol content. Cholesterol content in RBC of CHOL GP increased sharply within 1 to 3 days. Plasma cholesterol also increased during this period. After the first week, only small increases in both RBC and plasma cholesterol content were observed until the onset of the anemia several weeks later (Table I). In control guinea pigs (CONT GP), cholesterol content of RBC and plasma remained

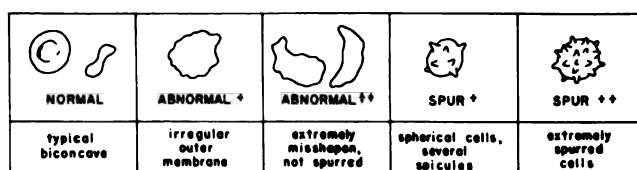


Fig. 1. Morphological classifications of red cells. Morphology of erythrocytes was assessed in whole blood obtained from the ear vein. One drop of blood was diluted in Hayem's solution, photographed using phase contrast optics, and counted by class.

TABLE I. SEQUENTIAL CHANGES OF PLASMA AND ERYTHROCYTE CHOLESTEROL CONTENT AND OF ERYTHROCYTE MORPHOLOGY DURING 15 WEEKS OF CHOLESTEROL FEEDING.

		Days								
		-1	+1	+3	+5	+7	+13	+21	+35	+85
Cholesterol ^a (mg/100 ml)										
RBC										
Expt. 1 ^b	CONT	121(7) ^x	141(3) ^y	114(3) ^y	123(3) ^y	141(2)		124(5)	126(5)	135(3)
	CHOL	123(9)	159(4) ^y	*145(5) ^y	*185(4) ^y	*181(5)		*193(8)	*201(8)	*283(8)
Expt. 2	CONT	159(4)	164(4)		165(4)		149(3)		119(4)	146(4)
	CHOL	160(4)	*191(4) ^y		*214(4) ^y		173(2) ^y		*240(4) ^y	
Plasma										
Expt. 1	CONT	37(7)	48(3)	47(3)	47(3)	64(2)		60(5)	50(4)	53(3)
	CHOL	*58(9)	*67(4)	*75(5) ^y	*189(4) ^y	*214(5)		*191(8)	*193(8)	*206(8)
Expt. 2	CONT	47(4)	47(4)		57(4)					
	CHOL	59(4)	*101(4) ^y		-					
Morphology ^c										
Expt. 1	CHOL									
	Abnormal (%)	11(5)	30(5)	17(5)	25(5)	36(5)		38(5)	24(5)	20(5)
Expt. 2	CHOL									
	Abnormal (%)	14(2)	30(4)		27(4)		74(4)		39(4)	
Expt. 2	Spur (%)	0	0	1	3	5		48	66	56
	Spur (%)	0	0		0		9		60	

^a Cholesterol content of erythrocytes (RBC) and plasma was determined at intervals after initiation of the cholesterol-containing diet in control (CONT) and cholesterol-fed (CHOL) guinea pigs. In experiment 1, blood was sampled by closed heart puncture with EDTA as anticoagulant; in experiment 2, it was sampled by ear vein with heparin as anticoagulant.

^b Two different subgroups were bled on Days 1 and 5 or 3 and 7 in order to avoid undue stress and blood loss.

^c Mean (number of animals); x, CHOL is different from CONT at $p < 0.02$; y, mean is different from preceding time point at $p < 0.02$.

^d Morphology of Hayem's-fixed red cells from whole blood of CHOL GP (see Materials and Methods). For classification, see Fig. 1. Abnormal, Ab⁺ plus Ab⁺⁺; Spur, Spur⁺ plus Spur⁺⁺. Mean (number of animals) of fractions of cells in each class. Total number of cells on each slide was 30-100 cells. The range of values for cells from CONT GP were as follows: % normal, 70-90; % Ab⁺, 6-20; % Ab⁺⁺, 0.8; % Spur⁺, 0.5; % Spur⁺⁺, 0.

unchanged during the experimental period with some fluctuations in that of the RBC.

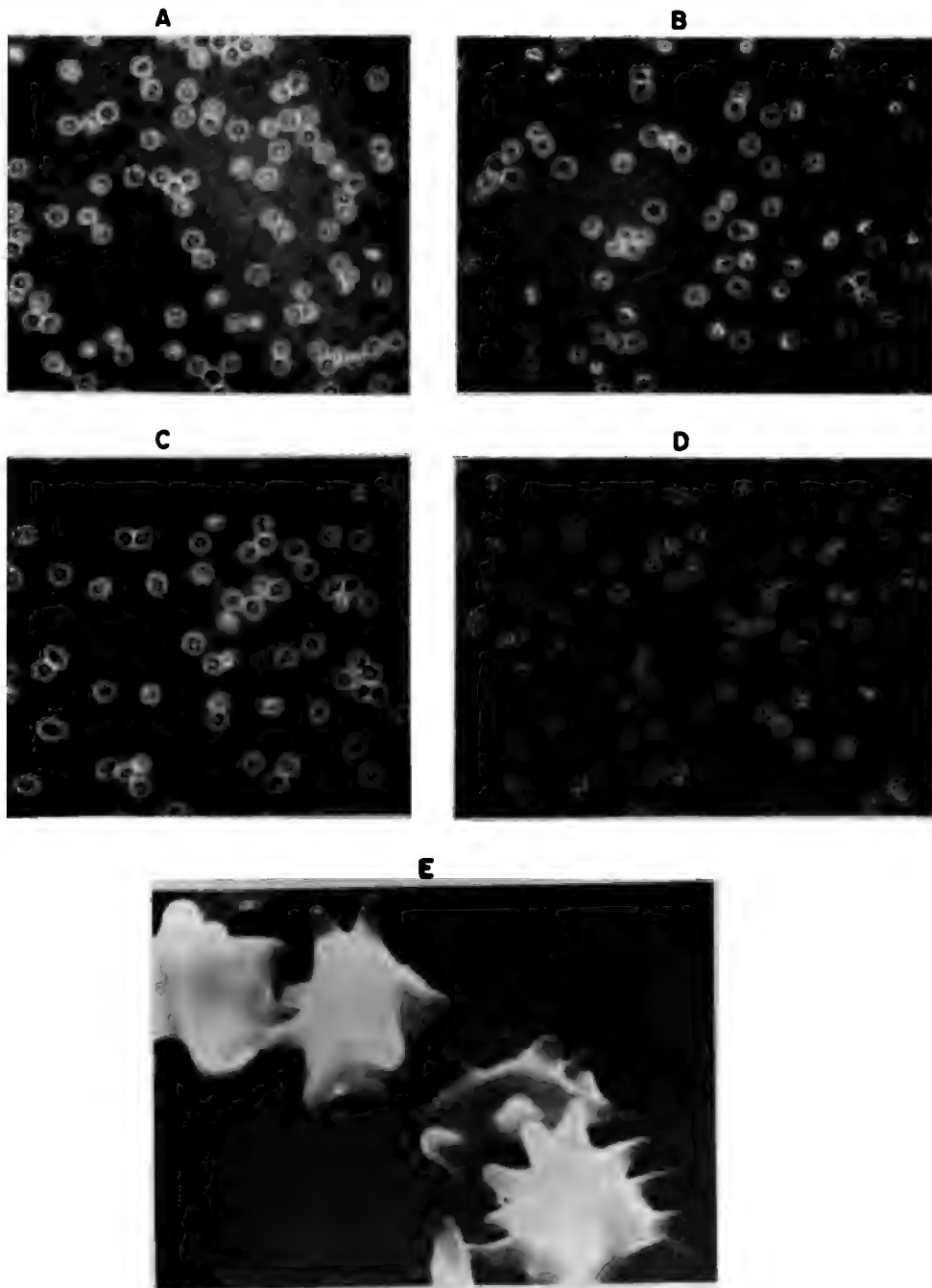
Increased RBC cholesterol content was associated with changes in morphology (Fig. 2). After 1 day of dietary cholesterol, 30% of the cells were characterized by wavy perimeters (Ab⁺ and Ab⁺⁺; Fig. 1; Table I). Significant spurring occurred first between Weeks 1 and 3 despite only minor additional increases of the membrane cholesterol content. A maximum of 65% echinocytes was observed at Week 5.

Osmotic fragility and cholesterol content. Osmotic fragility of RBC decreased simultaneously with increases of membrane cholesterol content. After 1 day of feeding chole-

sterol, osmotic fragility of CHOL RBC was significantly less than that of CONT RBC ($P < 0.01$) and decreased further to Day 5 (Fig. 3).

Energy metabolism and cholesterol content. ATP content of RBC and activities of ATPases, PK, Hex, and PFK, were found to be correlated with the percentage of reticulocytes ($r = 0.82-0.95$), and were not correlated with either the cholesterol content nor the morphological abnormalities.

Hemolysis, morphology, and cholesterol content. Red cell counts began to fall and the percentage of reticulocytes started to rise from 5-9 weeks after the start of cholesterol supplementation. The overt anemia



Electronmicrographs of guinea pig red cells. (A-D) Red cells of guinea pigs fed cholesterol for -1, and +21 days, respectively. Whole blood was fixed in Hayem's solution. Polaroid photographs using trast optics ($\times 280$). (E) Red cells of a cholesterol-fed, anemic guinea pig. Scanning electronmicrographs

(RBC count less than 3.5×10^6 cell/mm³) generally occurred 4–6 weeks later (Table II). The time varied between animals and between different groups of these outbred guinea pigs. The number of reticulocytes in CONT red cell populations was consistently less than 1% and the RBC counts averaged between 4.9 and 5.8×10^6 cells/mm³. This

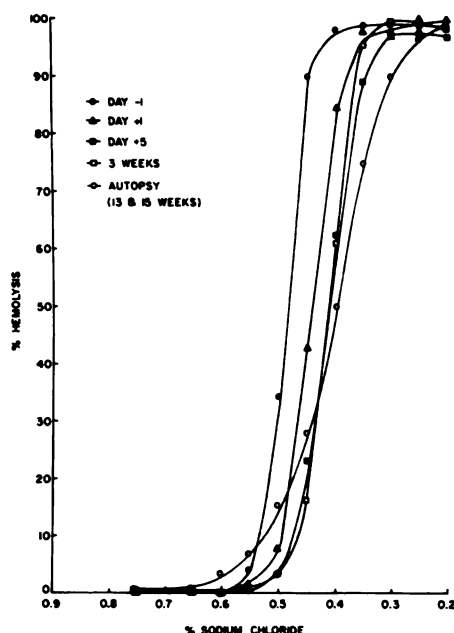


FIG. 3. Osmotic fragility of RBC from guinea pigs fed 1% cholesterol for different times as indicated (see Materials and Methods). Points are means of four to five animals. The concentration of NaCl for 50% hemolysis was 0.50, 0.44, 0.44, 0.41, 0.41, 0.41, 0.40, and 0.40% for Days -1, +1, +3, +5, +7, +21, and Weeks 5 and 13, respectively. The corresponding values for CONT cells were 0.50, 0.49, 0.47, 0.49, 0.48, 0.47, 0.47, and 0.47%. Differences between CONT and CHOL cells were statistically different at $P < 0.01$ from Day +1 on.

time course for the development of the hemolytic anemia is in contrast to the very early changes is red cell cholesterol content, osmotic fragility, and morphological abnormalities.

Discussion. We have previously reported a doubling of the cholesterol content of erythrocytes in cholesterol-fed anemic guinea pigs (1, 2). In the present study, red cell cholesterol was shown to increase within 1–3 days after supplementation of the diet with 1% cholesterol simultaneously with the increase of plasma cholesterol content. The rate of cholesterol accumulation by the red cells remained parallel to the increases in plasma cholesterol throughout the experimental period.

Morphological alterations and elevations of membrane cholesterol similar to those observed in CHOL GP can be produced *in vitro* when RBC from CONT GP are incubated in plasma from CHOL GP (6) and when human RBC are incubated in plasma from patients with spur cell anemia or with cholesterol-rich lipid dispersions (7). Our evidence, as well as that of others, indicates that the factor or factors in these plasma responsible for the net transfer of CHOL to the RBC and for the subsequent morphological abnormalities are abnormal lipoproteins with a high CHOL/PL ratio (4–7).

The appearance of Ab⁺ and Ab⁺⁺ forms occurred simultaneously with the initial increase of red cell cholesterol. Spur⁺ and spur⁺⁺ forms appeared about 2 weeks later, in the absence of further substantial increases of red cell cholesterol content. These abnormal RBC resemble the crenated disk-crenated spheres and the discocyte-echinocyte transformations described earlier (15, 16). If we assume that the

TABLE II. RED CELL COUNTS AND PERCENTAGES OF RETICULOCYTES IN CHOLESTEROL-FED GUINEA PIGS.^a

	Expt. 1						Expt. 2			
Weeks	1	3	5	9	12		3	5	7	9
RBC per mm ³ × 10 ⁶	5.2	5.8	5.4	4.8	3.5		4.9	4.7	3.6	3.5
Reticulocytes %			0.8	4.1	10.0		1.2	1.5	15.7	18.5
	Expt. 3			Expt. 4						
Weeks	9	11	13	5	11	14				
RBC per mm ³ × 10 ⁶	4.8	4.6	3.5	5.9	3.6	3.6				
Reticulocyte	4.1	6.6	9.3		16.7	22.6				

^a Red cell (RBC) and reticulocyte counts in guinea pigs when fed a 1% cholesterol-containing diet for varying periods of time in our replicate experiments are given; mean of four to six animals. Red cell counts in control guinea pigs averages 4.9 – 5.8×10^6 cells per mm³ whole blood. Reticulocytes were less than 1%.

in vivo are analogous to those observed *in vitro*, we could conclude that spiculation in the cholesterol-fed GP is associated with an increased cholesterol content of the red cell membrane and is the result of a series of sequential shape transformations. Proof of this hypothesis must await the demonstration that the Δb^+ , Δb^{++} , spur $^+$, and spur $^{++}$ are in transformations of the same cell.

The morphological abnormalities of the cholesterol-loaded GP RBC do not seem to be related to changes in the activities of key enzymes necessary for the energy metabolism of the cell nor to a depletion of ATP (8), changes which we did observe occur long after the appearance of the reticulocytes and were correlated with the erythrocytosis. Reticulocytes and young red cells have been reported to have higher concentrations of ATP (17) and increased activity of glycolytic enzymes (18) than do mature RBC.

The inverse association of osmotic fragility and cholesterol content has been reported to occur in patients with alcoholic liver disease and has been shown to be related to an increase of surface area (7, 10). Calculated surface area of the CHOL RBC from the critical hemolytic volume (19) and on the basis of an unchanged MCV of CHOL RBC indicate an increase of 4–8%, corresponding with the percentage increases of cholesterol ($r = 0.89$).

The increased osmotic fragility, possibly resulting from the increased local viscosity of cholesterol-loaded RBC (20) as well as to the decreased surface area, might lead to decreased RBC deformability, thereby increasing membrane fragmentation and sequestration of red cells.

Spiculated RBC might also be expected to decrease the ease of red cell filtration through the splenic microcirculatory system. However, it is unlikely that either the reduced osmotic fragility or spiculation of the cholesterol-loaded RBC contributes to the eventual appearance of the hemolytic anemia. Although a major proportion of GP RBC are severely spiculated after 3 weeks of cholesterol, the overt hemolytic anemia did not occur until an additional 6 to 9 weeks of cholesterol supple-

mentation. We have shown previously that the earliest signs of an anemia as indicated by bone marrow response, hematological parameters, and decrease of mean survival time of RBC appear at about Week 7 (2). Furthermore, splenectomy either before, during, or after initiation of the cholesterol-containing diet has no effect on the development of the anemia (21; W. Yamanaka and R. Ostwald, unpublished observations). Some other mechanism must be involved in the eventual development of the hemolytic anemia in cholesterol-fed guinea pigs.

Summary. We have studied the sequential changes of cholesterol content and morphology in RBC of cholesterol-fed GP and the effects of these changes on their energy metabolism and osmotic fragility. The results suggest that (i) spurring of GP RBC is not accompanied by obvious alterations in their energy metabolism and may be the end result of sequential shape transformations associated with cholesterol-loading, and (ii) neither the morphological abnormalities nor the decreased osmotic fragility of cholesterol-loaded RBC is directly related to the development of the hemolytic anemia.

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Interferon Assay of High Sensitivity (39580)

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There are many conditions where the ability to quantify low levels of interferon is important. For example, in the study of new compounds as interferon inducers, promises of compounds might be overlooked because the particular member of a group studied showed only minimal activity. The interrelationship of low levels of interferon and the immune response (1) is an area where a sensitive procedure would be useful. In situations where only small volumes of biological samples are available, with a sensitive assay it would be possible to dilute the small volume sufficiently to have a workable quantity of test material.

The present report summarizes experiments which have led to the development of a new interferon assay method that is significantly more sensitive than the one previously used in this laboratory. Through this assay, interferon has been quantitated in mouse and primate serums where other methods failed to detect any.

Materials and methods. Interferons. Human and mouse standard interferons No. G-537 and No. G-002-904-511 (Sigma catalog of research reagents, 1975) were used. Serum from mice treated with low molecular weight inducer S,2-hylisothiuronium 2HBr (AET) was used as a source of low titer interferon (L. Vovsky, H. Levy, D. C. Doherty, and others, in preparation).

Cell cultures. Human diploid fibroblast HFS-1 (Biofluids, Rockville, Md.) and L-203 (HEM Research, Inc.) and L-929 cells were used for interferon assay. Cell suspensions, at a concentration of $\times 10^4$ in 0.1 ml of Eagle's minimal medium (MEM) with 10% fetal bovine serum (FBS), were added to each well of 96 well microtiter plates (Falcon Plasticware). Cell cultures were incubated in a humidified atmosphere at 37°. Procedures for pre-

paring the cell cultures for interferon assays are described in the section of results.

Interferon assays. Serial 0.5 log dilutions of interferon were made in MEM with 2% FBS, and 0.1 ml of each dilution was added to quadruplicate cell cultures. After overnight incubation, the fluid was removed and the cells were challenged with 10^2 TCID₅₀/0.1 ml of Indiana strain of vesicular stomatitis virus (VSV) in MEM with 2% FBS. CPE was read 24-36 hr later. The degree of CPE was graded from 0 to 4, the latter being complete destruction of the cell sheet. The dilution of interferon that gave 50% protection ($2 + CPE$) was taken at the end point. Alternatively, reduction of virus hemagglutinin (HA) yield in interferon-treated cultures was used with Sindbis virus as the challenge for human cells (HFS-1 and HR-203) and GD-7 for mouse cells (L-929).

Results. Four different incubation conditions were used to prepare the cell cultures for assay. After the cells reached confluency at 37°, the first group of cultures (both human and mouse) were kept 24 hr in an incubator at 37°. The third group of confluent culture was aged at 30° for 5 days. The second and the fourth groups were the same cultures as the first and the third, but 24 hr before assay the medium was replaced with fresh MEM-10% FBS. The cells were further incubated at 37°. Table I summarizes data from the experiments. The first line, incubation for 24 hr at 37° after reaching confluency, is the most common method of growing cells for interferon assay (reference method). Assay of standard human and mouse interferons in these cultures showed that the titer of the reference interferon was in agreement with its nominal value, i.e., 4.2 ± 0.44 log units for human and 3.9 ± 0.35 log units for mouse interferon. Changing of the media in these cells did not increase the interferon titers. Some insignificant increase of interferon titers has

TABLE I. INTERFERON YIELD FOLLOWING DIFFERENT CONDITIONS OF AGING OF CELL CULTURES.

Aging conditions before interferon assay		Interferon titer (log units/ml)	
		Human interferon	Mouse interferon
1	(a) 37° for 48 hr, Reference method	4.2 ± 0.44	3.9 ± 0.35
2	(a) 37° for 24 hr	4.2 ± 0.5	3.9 ± 0.35
	(b) Change of medium		
	(c) 37° for 24 hr		
3	(a) 37° for 24 hr	4.6 ± 0.44	4.1 ± 0.5
	(b) 30° for 6 days		
4	(a) 37° for 24 hr	5.4 ± 0.44*	4.9 ± 0.23*
	(b) 30° for 5 days		
	(c) Change medium		
	(d) 37° for 24 hr		

* The difference between numbers presented on lines 1 and 4 are highly significant ($P < 0.001$ in Student's *t* test).

been found in cultures aged at 30° for 6 days: 0.4 log for human and 0.2 log for mouse standard interferons. However, changing of the medium on the cell cultures aged at 30° significantly increased the titers of both human (from 16- to 30-fold) and mouse interferon (up to 10-fold).

The substantial increase in the sensitivity of the interferon assay was an important factor in finding a new class of low molecular weight interferon inducers: mercaptoalkylamines and derived thiophosphates. Table II contains data from an experiment where three samples of serum from mice treated with one such compound, AET, as well as two samples of mouse standard interferon, were assayed in cell cultures using the new technique. With the standard test, no interferon was detected in the sera from AET-treated mice. However, using procedure no. 4 (see Table I), significant levels of interferon were found. Since undiluted serum was toxic to the cells, a 1:10 dilution was used as the lowest concentration. Since no interferon was detected at this dilution using the reference method, one cannot state the exact degree of increase in sensitivity found in this sample.

Discussion. The data presented here summarize minor modifications of commonly used interferon assay procedures which have led to an increase from 16- to 30-fold in the sensitivity in human interferon assays and up to 10-fold in mouse interferon. The effectiveness of aging cells at 37° previously has been reported to increase by 1.6- to 3-fold (2-5). However, the new combination of procedures currently presented, further

TABLE II. DETECTION OF INTERFERON BY THE AGING METHOD.

Interferon	Samples number	Interferon titer ^a as measured in L cells incubated under different conditions	
		Reference method	Aging method
Mouse serum interferon induced by ip administration of 2 mg of AET per mouse	1	<1.0 ^b	1.0
	2	<1.0	1.9
	3	<1.0	1.7
Normal mouse serum	4	<1.0	<1.0
Reference standard mouse interferon	5	4.2	5.3
	6	4.1	5.1

^a Log units per milliliter.

^b For assay of serum or tissue interferons, a starting dilution of 1:10 was used to avoid toxicity.

increases the sensitivity of interferon assay in a simple and reproducible way.

We have used this method to detect interferon-inducing activity in different groups of weak interferon inducers under conditions where the standard method of assay gave dubious results. It is realized, of course, that when one is working with high concentrations of interferon an increase in the sensitivity of an assay has no particular merit. However, even with samples of moderate activity, where the volume of the samples is extremely small a sensitive assay method can serve a useful purpose. This method might also be useful in studying human and animal specimens for minimal concentrations of interferon.

Summary. A method of aging cells is described which allows for an increase in sensitivity of the assay of interferon. After reaching confluency the cell cultures are aged at 30° for 5 days, the growth medium is replenished, and the cells are incubated for an additional 24 hr at 37° before applying interferon. Using cell cultures so treated (diploid cells HFS-1 or HR-203), and mouse L-929 cells), the sensitivity of the interferon assay increase from 16- to 30-fold for human and up to 10-fold for mouse standard interferon.

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Effects of Corticosterone Treatment on Puberty in Female Rats (39581)

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Adrenal status has been shown to play a role in the timing of puberty onset (1). Adrenalectomy prior to 26 days of age delays both vaginal opening and ovulation (2, 3), while later adrenalectomy diminishes the ovarian weight response to gonadotrophin (4) and the number of ova shed at the first ovulation (5). Excessive adrenal activation due to adrenocorticotrophin (ACTH) administration is also associated with delayed puberty (6). We have recently noted that stimuli that altered the timing of the corticosterone rhythm in the blood were associated with changes in puberty onset in immature mice (7). Most studies of the effects of excessive corticoids on puberty have involved stressful routes of administration such as daily injections or pellet implants. Since the stress itself might have altered the secretion of other pituitary hormones (8, 9), another route of administration seemed desirable. To this end, corticosterone dissolved in the drinking water and available *ad libitum* was provided as a nonstressful source of steroid in order to determine what effects exogenous corticosterone would have upon puberty onset in intact rats.

Materials and methods. Female Sprague-Dawley derived rats were obtained at 21 days of age from Sasco Laboratories, Omaha, Nebr., and were housed three per cage in hanging stainless steel cages in controlled light (lights on 0500 hr and off 1700 hr) and temperature $21 \pm 1.2^\circ$ [SE]. The day after they arrived in the lab, the rats began to receive corticosterone in their drinking water (16, 24, 160, and 240 $\mu\text{g}/\text{ml}$) provided *ad libitum* as a solution of 4% ethanol in tap water. Controls received 4% ethanol in tap water, tap water only, or 0.9% physiological saline. The rats were weighed daily between 0800 and 1000 hr and vaginal smears by lavage were started on the day of vaginal opening. Laparotomy was performed on the day of the first estrous smear (solely cornified cell), and ovulation

was confirmed by the presence of hemorrhagic follicles and a dilated oviductal ampulla. All animals examined in this way were found to have ovulated. Rats that did not ovulate were killed at 60 days of age. To determine the blood levels of corticosterone produced by the available corticoid in the drinking water, 22-day-old rats were given various concentrations of corticosterone (Sigma) and killed by decapitation on the fourth day after the start of steroid administration at 0800, 1600, or 2300 hr of colony time. Corticosterone in the serum was measured by a fluorometric procedure previously described (10). The experiment was performed in November when puberty in our colony is later than in summer (2).

Results. In controls maintained on tap water, vaginal opening took place at 39.0 ± 2.2 days and ovulation within 2 days (41.8 ± 2.6 SE) (Table I). Puberty occurred at practically the same time in rats given 4% ethanol in tap water to drink. The lowest concentration of corticosterone (16 $\mu\text{g}/\text{ml}$ of 4% ethanol in tap water) did not alter the time of vaginal opening or ovulation. The next concentration (24 μg) did not significantly delay vaginal opening but did delay ovulation. The two high concentrations of corticosterone (160 and 240 μg) delayed both vaginal opening and ovulation ($p = 0.02$). At the highest concentration (240 μg) ovulation had occurred in only one rat by 60 days of age when the experiment was terminated. The other animals had undergone vaginal opening but retained infantile uteri (92.4 ± 14.5 mg; Table I) and ovaries with primarily small follicles.

The average daily water consumption increased from 10 ml/day to approximately 20 ml/day in all groups during the course of the experiment.

Rats given 24 μg of corticosterone/ml of drinking water grew at normal rates (inferred from body weight data collected at 2-day intervals). Their body weight at ovula-

TABLE I. INDICES OF PUBERTY IN INTACT RATS GIVEN CORTICOSTERONE DAILY IN THEIR DRINKING WATER.^a

Treatment	Number of rats	Age at		Body weight at ovulation	Organ weights on day of ovulation		
		Vaginal opening	Ovulation		Ovaries	Uterus	Adrenals
	6	39.0 ± 2.2	41.8 ± 2.6	128.6 ± 6.5	35.3 ± 3.6	163.8 ± 5.4	32.6 ± 5.4
%	7	39.3 ± 1.7	40.7 ± 2.2	136.0 ± 6.6	44.5 ± 2.8	206.3 ± 16.9	32.2 ± 2.5
corticoster-	7	39.6 ± 1.8	41.6 ± 1.7	129.0 ± 5.4	36.9 ± 2.3	178.8 ± 13.0	33.8 ± 2.4
corticoster-	10	42.9 ± 1.6	<u>49.8 ± 2.3</u>	<u>152.7 ± 7.7</u>	46.6 ± 3.5	199.9 ± 19.4	36.9 ± 3.1
corticoster-	9	<u>46.3 ± 2.4</u>	<u>49.5 ± 2.5</u>	120.0 ± 9.6	39.0 ± 3.9	200.9 ± 30.8	<u>14.6 ± 2.0</u>
Corticoster-	10	<u>51.2 ± 4.7</u>	<u>> 60 days</u>	92.1 ± 4.8 ^b	<u>25.9 ± 3.1^b</u>	<u>92.4 ± 14.5^b</u>	<u>15.1 ± 1.3^b</u>

expressed as mean ± SE. Underlined values different from intact controls given 4% ethanol in tap water ($p < 0.05$), Student's t test, nonpaired

weights at 60 days of age since ovulation did not occur. Concentrations of corticosterone (16 μg , etc.) refer to micrograms per milliliter in 4% tap water. All treatments were begun at 22 days of age.

as heavier than that of controls maintained on 4% ethanol in tap water ($p < 0.01$, Student's t test, nonpaired variance). Corticosterone (160 μg) did retard weight gain for a few days, but body weights in these rats at vaginal opening and ovulation were not significantly different from controls.

The normal pattern of serum corticosterone in our 14-hr light, 10-hr dark cycle was of a peak just before dark onset (10 hr sample time). Rats maintained on tap water, 0.9% saline, or 4% ethanol in tap water, showed the usual 1600-hr peak. Rats given the two lower concentrations of corticosterone did not have the 1600-hr peak and analysis of variance failed to reveal a significant difference at 0800, 1600, and 2300 hr of colony time. Rats given the two higher concentrations of corticosterone had lower corticosterone values at 1600 hr than normal ($p = 0.05$) and elevated corticosterone at 0800 and 2300 hr ($p = 0.05$).

Discussion. Three results emerge from this study. First, despite the suppression of the usual late afternoon corticosterone peak, puberty as expressed in vaginal opening and the time of the first ovulation occurred at a normal age in rats given the

lowest concentration (16 $\mu\text{g}/\text{ml}$) of exogenous steroid in their drinking water. Second, vaginal opening and ovulation were dissociated in rats given 24 μg of corticosterone/ml despite the normal rate of weight gain. Third, at higher doses of exogenous steroid, the daily rhythm of serum corticosterone was altered and perhaps inverted while both vaginal opening and ovulation were retarded. In these rats growth was also retarded.

If the 24 μg of corticosterone treatment had not been included, it would be appropriate to attribute the puberty-delaying effects of exogenous steroid to growth retardation. The association between metabolic status, growth, and sexual maturation has been developed by Frisch and Revelle (11) who have proposed a critical weight hypothesis for puberty in humans. According to this concept, puberty takes place when an individual reaches a threshold growth or metabolic status. Growth retardation due to underfeeding will dissociate vaginal opening and ovulation in rats (12). The dissociation between vaginal opening and ovulation in the presence of a normal growth rate in rats given 24 μg of corticosterone suggests that another factor may be operating as well. Corticoids act as estrogen antagonists in the uterus (13) and it is possible that the sensitivity of vaginal tissue to this effect is less than that of the uterus, allowing for estrogenic stimulation of vaginal opening in the absence of ovulation. We have not yet measured serum estrogens or progestins in these

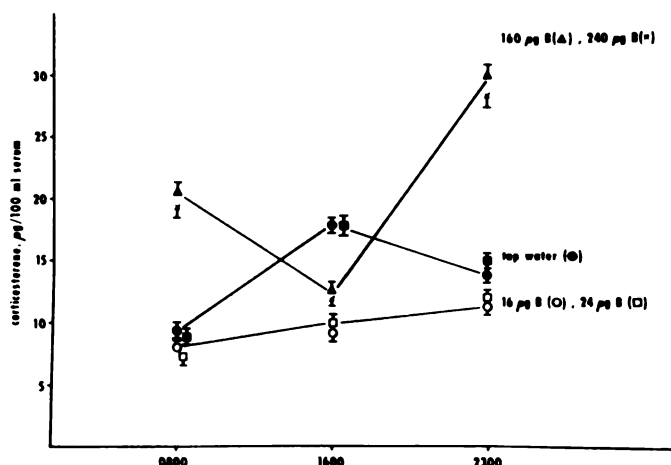


FIG. 1. Effects of corticosterone in the drinking water upon serum corticosterone levels. Rats were given various concentrations of corticosterone dissolved in 4% ethanol in tap water *ad libitum* for 3 days beginning at 22 days of age, and samples were collected by decapitation on the fourth day (at 25 days of age). Controls received either tap water or 4% ethanol in tap water (●). The results of the control groups are combined. The experiment was done at the same time as the study represented in Table I on litter mates or the animals in that experiment. Data expressed as mean \pm SE, six rats per group. The time of day at autopsy is shown on the abscissa.

animals to determine whether they are normal or diminished. The exogenous steroid could be acting at any point in the gonadal system to diminish target organ sensitivity or steroid secretion. Corticoids do alter pituitary sensitivity to releasing hormones and gonadal sensitivity to gonadotrophins [reviewed in (14)].

The other key finding is that normal puberty can occur in rats with disrupted adrenal rhythmicity as a result of very low dose exogenous corticosterone (16 μ g/ml of tap water), lending support to our previous indications that the adrenal rhythm itself is not required for normal puberty onset although adrenal steroids must be present (15).

The growth retardation produced by higher doses of corticosterone was associated with retarded sexual maturation. This finding supports the Frisch and Revelle hypothesis (11).

In conclusion, two results of this study cast some light on the process of puberty onset. First, the presence of a normal adrenal rhythm is not a requirement for normal vaginal opening and ovulation. Second, higher doses of corticosterone (24 μ g) can cause a dissociation between vaginal opening and ovulation without resulting in significant growth retardation. It is possible that

these levels mimic the effects of chronic stress which can also lead to a separation between vaginal opening and ovulation (unpublished data). The response to the 24- μ g dose of corticosterone clearly suggests that the effect of excess corticosterone is not mediated solely by interference with normal growth rates.

Summary. To study the role of excess corticosterone on regulating puberty onset without the added component of a stressful route of administration, corticosterone was made available *ad libitum* in the drinking water beginning at weaning age (22 days of age). The lowest dose (16 μ g of corticosterone/ml of 4% ethanol in tap water) did not delay puberty but did suppress the normal peak of corticosterone at 1600 hr. The next dose (24 μ g) also suppressed the evening corticosterone peak. In these rats, vaginal opening was normal but ovulation was delayed in comparison to controls given 4% ethanol in tap water or tap water alone. Growth rates in the rats given 16 or 24 μ g were normal. The two high concentrations (160 and 240 μ g of corticosterone/ml 4% ethanol of in tap water) delayed both vaginal opening and ovulation, produced high levels of corticosterone in the blood at 0800 and 2300 hr and a depression of corticoster-

one at 1600 hr, and were associated with significant growth retardation. It is concluded that a normal adrenal rhythm is not required for puberty onset and that the ability of exogenous corticosterone to delay puberty onset can be mediated through routes other than growth retardation alone.

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Alterations in Thyroidal Calcitonin Content of Rats Fed Diets of Varying Ca:P Ratios^{1,2} (39582)

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It is well established that secretion of calcitonin (CT) from the mammalian thyroid gland is strongly influenced by the calcium concentration of the serum and is stimulated by hypercalcemia (1). In most mammalian species, thyroidal stores of CT are relatively large with respect to rates of secretion (1, 2). However, large changes in thyroid gland content of CT can be produced in the rat by inducing a sustained hyper- or hypocalcemia. Chronic hypercalcemia produces depletion of thyroidal CT content whereas chronic hypocalcemia following parathyroidectomy is associated with a progressive increase in CT content (3).

Since it is well known that changes in the dietary Ca:P ratio can influence the concentration of blood calcium (4-8), we initiated the present study to examine possible effects of alterations in the dietary Ca:P ratios on the content of CT in the rat thyroid gland. Parathyroidectomized (parex) and adrenalectomized (adrex) rats, as well as intact rats, were studied. The results show that alterations in the dietary Ca:P ratio produce large changes in thyroidal CT and that these changes cannot be explained simply on the basis of chronic changes produced in the serum levels of calcium.

Materials and methods. Male Holtzman rats (Madison, Wisc.) were grouped so that

their mean body weight at the beginning of the study was the same. Experimental diets were prepared as previously described (7, 8) and rats were pair-fed for 1 to 2 months. Dietary ratios of Ca:P were varied between 1:8 and 8:1 either by changing the calcium content and keeping the phosphorus content constant at 0.4% (7) or by changing the phosphorus content and keeping the calcium content constant at 0.4% (8). Six rats from each group were used for serum analyses, and for the assay of thyroidal CT, glands from two to three rats in each group were pooled for analysis.

Surgery was performed under ether anesthesia. Some rats were bilaterally adrenalectomized 7 days before the feeding experiments, and these were given 0.15 M NaCl in place of distilled drinking water during the experimental period. Parathyroidectomy was performed by surgical excision and only rats having serum calcium levels below 8 mg/100 ml and serum phosphorus levels above 11 mg/100 ml 1 week after surgery were used. In this study involving different surgical procedures and high phosphate diets (Table I), initially there were 10 rats per group. At the end of 1, 2, and 4 weeks, two animals from each group were killed by cardiac exsanguination under ether anesthesia, and determinations of serum calcium, phosphate, and CT were performed. The thyroid glands of both rats in each group were pooled and either analyzed immediately for CT or frozen rapidly on dry ice and kept at -20° until assayed. At the end of 8 weeks, the remainder of the animals were killed and similar analyses were performed. The number of animals remaining in each group at this time ranged from one to four. Since the results were similar at 1, 2, and 4 weeks, only the data obtained at the end of 4 and 8 weeks are given.

Serum calcium was determined by atomic

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reption spectrophotometry, and serum phosphorus, by a modification of the Fiske-Crow method (9). For CT assays, dried thyroid glands were analyzed for CT bioassay and radioimmunoassay. Bioassays with porcine CT as a reference standard were performed as previously described. Radioimmunoassays were conducted as described earlier (11) using highly purified rat CT for both radioiodination and as labeled reference standard. Statistical evaluation of differences in calcium and phosphate levels in sera was done by Student's *t* test.

Results. Analyses of CT content of thyroid glands. Figure 1 shows the CT content in thyroid extracts analyzed by both bioassay and radioimmunoassay. Values obtained by the two methods showed excellent correlation ($r = 0.86$, $n = 18$). The ratios obtained by dividing the bioassay value (milliunits per gland) by that for the radioimmunoassay (micrograms per gland) gave a mean of 271 \pm 5 mU/ μ g, a value which corresponds closely to the specific biologic activity of rat CT (11).

Effects of varying dietary Ca:P on thyroidal CT of intact rats. Figure 2 summarizes findings obtained in intact rats fed experimental diets varying in Ca:P for 4 weeks. In this experiment, Ca:P was varied by altering dietary Ca. No significant differences in body weight were found. However, as the dietary Ca:P ratio fell from 8:1 to 1:8, mean Ca decreased slightly while serum P increased. Thyroidal CT remained quite constant at ratios between 8:1 and 1:4, but with a ratio of 1:8 there was a marked elevation in thyroidal CT. Figure 3 shows results of a similar study in which the Ca:P ratio of the diet was altered by changing dietary P. Rats fed diets with Ca:P ratios of 1:8, 4:1, or 8:1 had very little weight gain. Although the patterns of change in serum Ca, serum P, and thyroidal CT were similar to those observed in the study shown in Fig. 2, changing the dietary Ca:P ratio by altering dietary phosphorus content of the diet appeared to have a greater effect on these variables. In this study, thyroidal CT rose progressively as the dietary Ca:P ratio fell below 4:1 and at a dietary Ca:P of 1:8, the levels of serum phosphorus were significantly higher ($P < 0.01$) and those of serum cal-

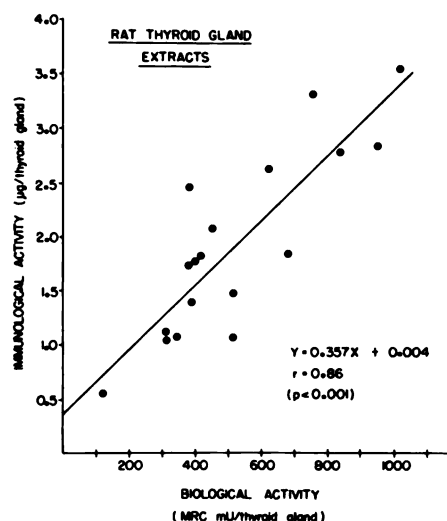


FIG. 1. Positive correlation between values for CT obtained by radioimmune assay and by bioassay. Various dosages of each thyroid gland extract (see Methods) were analyzed by both assay methods.

cium were significantly lower ($P < 0.01$) than when dietary calcium was varied. In both Figs. 2 and 3, the results indicate that thyroidal CT rose as (i) the dietary Ca:P ratio fell, (ii) the serum Ca fell, and (iii) the serum P rose. Detectable serum levels of CT were found only in rats fed the diet with Ca:P = 1:8.

Influences of parathyroidectomy and adrenalectomy on thyroidal CT. Table I shows alterations in serum Ca and thyroidal CT in parathyroidectomized and adrenalectomized rats fed diets having a Ca:P ratio of 2:1 (0.8% Ca and 0.4% P) or 1:8 (0.4% Ca and 3.2% P) for 4 or 8 weeks. Within each treatment group (intact, parathyroidectomized, or adrenalectomized), mean thyroidal CT was higher and mean serum Ca lower in rats fed a ratio of 1:8 compared to those fed diet with a ratio of 2:1. Overall, however, CT levels showed no significant correlation with serum Ca. For example, similarly high CT levels were observed in rats fed a ratio of 1:8 for 8 weeks regardless of their surgical treatment, despite the fact that their serum Ca levels were quite different. In general, thyroidal CT levels in each of the three groups appeared to be related to the length of time that the diet with the Ca:P ratio of 1:8 was fed, those on diet 8 weeks having a higher level than those fed the diet for only 4 weeks.

THYROIDAL CT AND DIETARY CA:P RATIOS

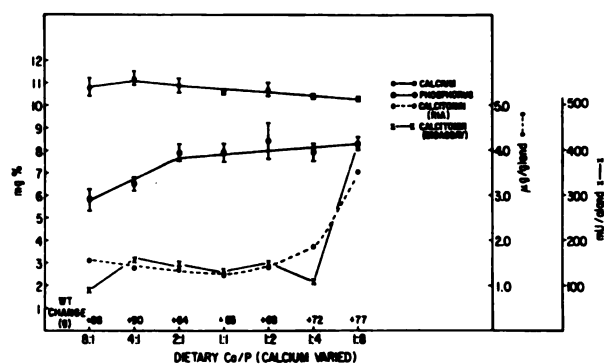


FIG. 2. Thyroidal CT as measured by radioimmunoassay (●—●) and bioassay (×—×), and the serum levels of calcium (●—●) and phosphorus (○—○) are plotted against the dietary Ca:P ratios shown on the abscissa. The vertical lines represent the standard deviation of the mean. Mean changes in body weight (grams are shown at the bottom of the graph.

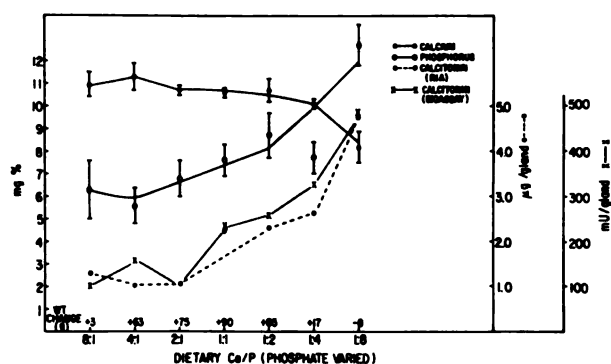


FIG. 3. Same legend as for Fig. 2.

TABLE I. CALCITONIN CONTENT OF THYROID GLANDS AND LEVELS OF SERUM CALCIUM IN RATS FED DIETS WITH VARYING Ca:P RATIOS.^a

Treatment	Dietary Ca:P (ratio)	Weeks on test	Body wt (g)	Serum		Radioim- mune assay (μg/gland)	Bioassay (mU/gland)
				Ca (mg/100 ml)	P		
Group 1							
Parex [3]	1:8	8	299	5.0	13.7	3.56	1010
Parex [2]	1:8	4	353	7.2	14.3	2.61	662
Parex [2]	2:1	8	417	7.9	8.6	1.07	315
Parex [2]	2:1	4	348	8.2	11.1	1.10	506
Group 2							
Intact [4]	1:8	8	336	8.8	13.4	2.79	832
Intact [2]	1:8	4	322	8.4	12.1	1.49	509
Intact [2]	2:1	8	418	9.6	5.8	1.77	398
Intact [3]	2:1	4	416	10.2	8.1	1.10	303
Group 3							
Adrex [3]	1:8	8	377	9.6	6.5	2.82	947
Adrex [2]	1:8	4	325	9.5	10.3	2.09	457
Adrex [4]	2:1	8	475	10.0	7.2	1.40	393
Adrex [2]	2:1	4	415	10.0	8.7	1.10	331

^a The diet with Ca:P = 1:8 contained 0.4% Ca and 3.2% P and that with Ca:P = 2:1 contained 0.8% Ca and 0.4% P. The numbers in brackets refer to the number of animals in each group.

Discussion. These findings clearly show that thyroidal stores of CT were altered by feeding rats diets which varied in their Ca:P ratios (Figs. 2 and 3; Table I). Furthermore, the results indicate that thyroidal CT content increased as the dietary Ca:P ratio was lowered irrespective of the blood calcium concentration which the animals were able to maintain.

It has been shown that CT levels in rat thyroid gland varied inversely with the existing levels of blood calcium in rats chronically maintained under conditions of hyper- or hypocalcemia and it was suggested that the increased CT content during hypocalcemia perhaps resulted from the absence of a stimulus for CT release in the presence of continued biosynthesis of CT (3). Conversely, decreased CT content with chronic hypercalcemia was presumed to be caused by continued high stimulus for release together with an inability of biosynthetic mechanisms to maintain normal thyroid levels of CT (3). In the present study, measurable amounts of CT in sera were found only in rats fed diets with Ca:P ratio of 1:8. This suggests that the capacity of the thyroid gland to store CT may have been exceeded resulting in its spillage into the serum. Our findings agree with those of Gittes *et al.* (3) in showing that CT levels in the thyroid gland increase as blood Ca falls. However, the present findings also indicate that the changes in thyroidal CT levels observed are not explained simply by changes in blood Ca. Rather, changes in CT levels in the thyroid in the present study appeared to be correlated more closely with the dietary Ca:P ratio and the length of time the diet was fed. This conclusion is supported by the finding that although parex rats fed the high phosphate diet had severe hypocalcemia (5.0 mg/100 ml) and the highest content of thyroidal CT, the adrex rats fed the same diet had almost the same thyroidal content of CT but were essentially normocalcemic (9.5 mg/100 ml). Although adrenalectomy had no effect on serum calcium or phosphate of rats fed diet with Ca:P = 2, of interest is the finding, for which we have no adequate explanation, that when adrenalectomized rats were fed diet with Ca:P = 1:8, the fall in serum calcium and rise in serum

phosphate that was observed in intact rats fed the same diet was prevented to a large extent. At the same time, no apparent differences were observed in thyroidal CT between intact and adrenalectomized rats fed this diet. Further studies will be required to clarify the mechanisms responsible for changes in thyroidal CT content and the factors responsible for the partial protection of adrex rats fed a Ca:P ratio of 1:8 against hypocalcemia and hyperphosphatemia but not against the increase in thyroidal CT.

Summary. The thyroidal content of CT was measured by bioassay and radioimmunoassay in intact, parathyroidectomized, and adrenalectomized rats fed diets with varying Ca:P ratios for 4 to 8 weeks. There was a good correlation between values obtained with the two assay methods. In intact, parex, and adrex rats, thyroidal CT content was higher 4 and 8 weeks after feeding a diet with a Ca:P ratio of 1:8. However, there was a little difference in overall CT content between intact, parex, and adrex rats despite marked differences in the levels of serum calcium. CT content appeared to be related to the length of time the rats were fed the high phosphate diet. In intact rats fed diets with Ca:P ratios ranging from 8:1 to 1:8 and prepared by varying the calcium content (P constant at 0.4%), the CT content of thyroid gland and serum was elevated only in those animals fed the 1:8 diet. In other intact rats fed with diets having the same range of ratios but prepared by varying the phosphorus content (Ca constant at 0.4%), thyroidal CT increased progressively with the diets having a Ca:P ratio below 1:1. The results indicate that changes in thyroidal CT stores produced by feeding diets with varying Ca:P ratios cannot be explained solely on the basis of changes in the levels of serum calcium.

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Effect of Cholecystokinin-Pancreozymin on Pancreatic Acinar Cells from Rats of Different Ages¹ (39583)

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In recent years, the gastrointestinal hormones gastrin and cholecystokinin-pancreozymin (CCK-PZ) have been looked upon as growth factors of the digestive tract. A survey on the spectrum of physiological responses affected by these hormones shows that chronic *in vivo* administration of large amounts of pentagastrin causes oxyntic gland hyperplasia (1). Berger and Johnson (2) conclude from studies on the ontogenic development of the small intestine, that some aspects are dependant on weaning and gastrin as the mediator.

Hormonal control of pancreatic growth could be controlled by gastrin and CCK-PZ. Indeed, in hypophysectomized rats, pentagastrin treatment stimulates pancreatic hyperplasia in addition to hypertrophy. In normal rats, large doses of pentagastrin also result in pancreatic acinar cell hyperplasia (4).

CCK-PZ and gastrin share the same terminal amino acids which comprise the C-terminus of the gastrin molecule, and thus expect the duodenal hormones to have comparable trophic effects on pancreatic tissue. Recent data from Barrowman and Guthrie (5) indicate a certain correlation of the trophic effect of CCK-PZ on pancreatic tissue since CCK-PZ octadecapeptide administration was associated with an increase in DNA synthesis in the pancreas and in the oxyntic gland or duodenum.

Barrowman *et al.* (6) established clearly that pentagastrin administration at a dose of 20 IVY units/kg twice daily for 5 days, was associated with increases in cell mass and cell number.

In similar studies, Barrowman and Guthrie (7) also established the trophic effect of CCK-PZ on the exocrine pancreas.

Since the trophic effect of an hormone implies the regulation of a number of growth-related processes in the target tissue, such as stimulation of protein, RNA, and DNA synthesis, these major effects should be seen at any time during the normal growth of an animal. Because all the previous studies on the pancreas were performed on adult animals, we decided to evaluate the trophic effect of CCK-PZ at different times during postnatal development of the rat exocrine pancreas.

Materials and methods. Sprague-Dawley rats from our own colony were used throughout these studies. The day of parturition was set as Day 1 and the number of rats per litter was fixed at 10 at that time. Half of them served as controls and were injected ip with saline while the other received CCK-PZ (20 IVY units/kg, twice a day) for 5 days before sacrifice. After an overnight fast, the rats were weighed and decapitated at the age of 11, 26, 47, 82 days (adult).

The whole pancreas was excised, trimmed of fat, weighed, and then homogenized in 4 M urea (75 mg/ml). Samples of the homogenate were precipitated in 2.1 N perchloric acid (PCA) for RNA and DNA determinations. Samples of 800 μ l were also centrifuged for 1 hr at 235,000g using a SW-50-1 rotor in a Beckman L2-65B; the supernatant was saved for amylase, lipase, chymotrypsin (Chti), and soluble proteins assays.

Amylase activity was assayed according to Bernfeld (8), lipase according to Charbonneau and Morisset (9), and chymotrypsin according to Hummel (10). Activities were expressed as total units in the gland and units/100 μ g of DNA, a unit being defined as micromoles of products liberated per minute.

RNA and DNA were extracted as described by Mainz *et al.* (6); DNA was deter-

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mined by the diphenylamine method using calf thymus DNA as a standard (11). RNA was determined by the orcinol method using yeast RNA as a standard (12), while protein was determined according to Lowry *et al.* (13).

Results. Figure 1 shows the effects of chronic administration of saline and CCK-PZ on body weight, pancreatic weight, pancreatic weight in terms of gram body weight, and pancreatic weight in terms of 100 μ g of DNA.

Body weights were not affected by CCK-PZ treatment for all the ages studied. Pancreatic weights remained unaffected at 11 days but were significantly increased by 20.5% at 26 days, by 23.2% at 47 days, and by 24.8% in the adults. Data on pancreatic weight expressed in terms of grams body weight indicate that CCK-PZ is associated with hyperplasia and or hypertrophy at all ages, but the effect is less pronounced at 11

days. However, the expression of pancreatic weights in terms of 100 μ g of DNA, which is indicative of cell mass, shows that CCK-PZ is efficient only at 26 days. The high increases in amylase, chymotrypsin, and soluble protein concentrations can explain the increase in cell mass which prevails even if DNA is also being stimulated.

As the DNA content is generally considered indicative of cell numbers, CCK-PZ is associated with significant increases of 16.4% in the 47 days and adult rats (Fig. 2). The hyperplastic effect was less important at 26 days (8.3%) but still significant while no effect was seen at 11 days. Since the body weight did not change in the course of treatment, the expression of DNA per gram body weight can represent the number of cells per pancreas. Data thus show that CCK-PZ increased significantly the number of pancreatic cells by 12.4% at 11 days, by 18% at 47 days and by 15% in the adults

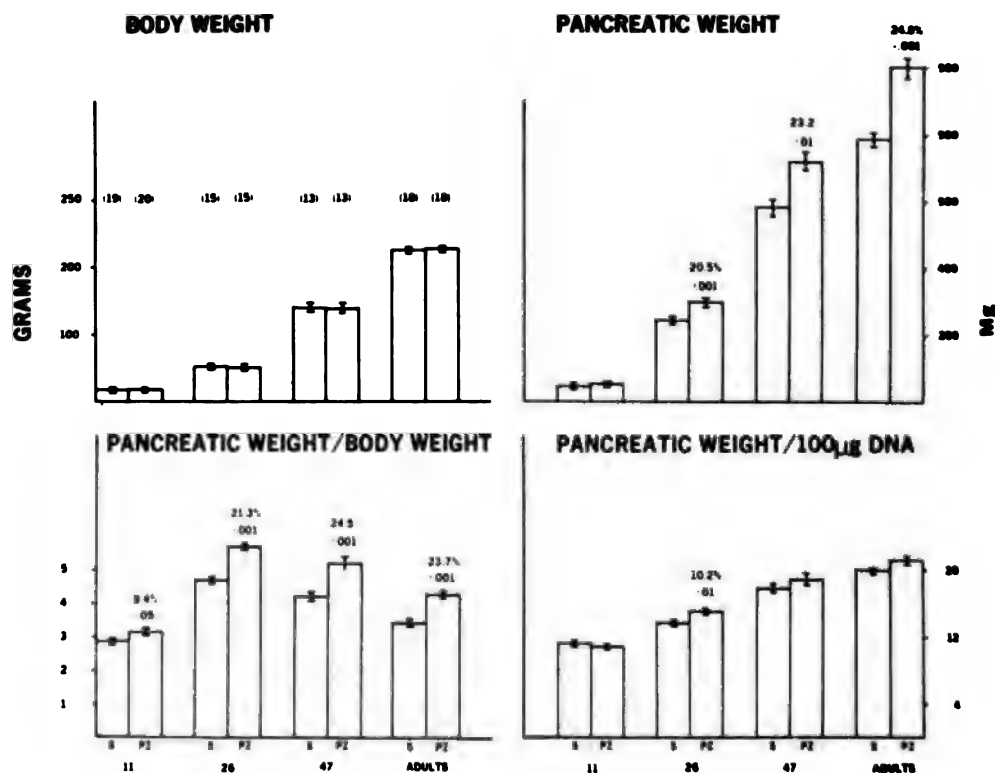


FIG. 1. Effects of chronic administration of cholecystokinin-pancreozymin on body weight, pancreatic weight, pancreatic weight/body weight, and pancreatic weight/100 μ g of DNA. (n): Number of animals in each group; S: saline group; PZ: cholecystokinin-pancreozymin group. Saline and CCK-PZ (20 IVY units/kg twice a day were injected ip for 5 days.

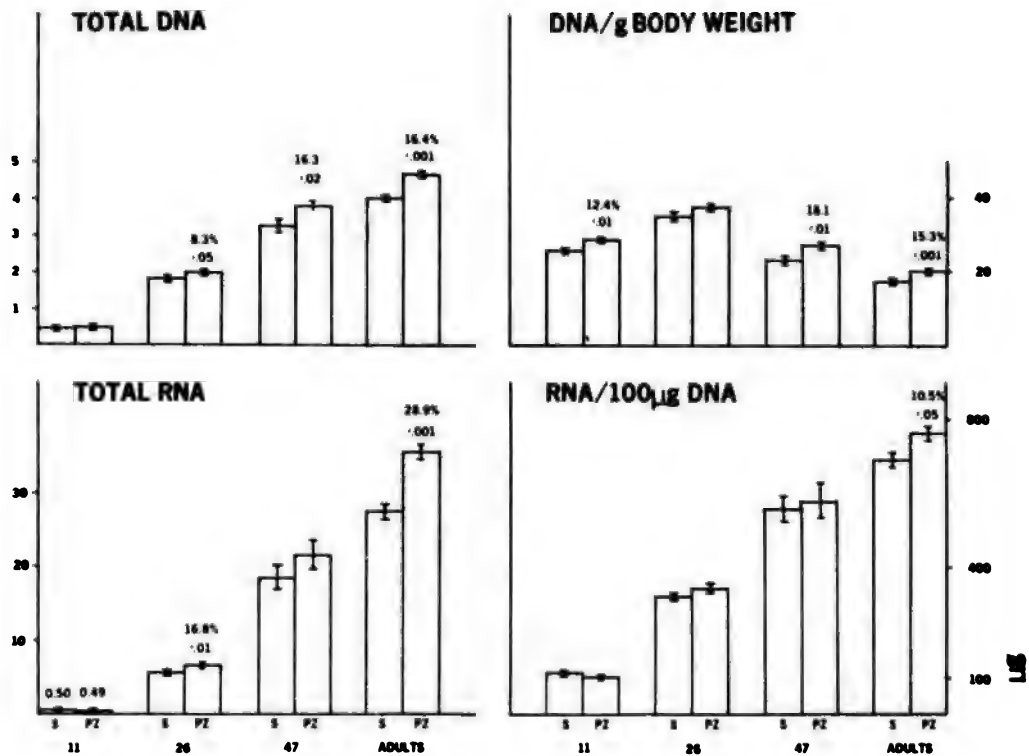


FIG. 2. Effects of chronic administration of cholecystokinin-pancreozymin on total pancreatic DNA content, DNA per gram body weight, total RNA, and its concentration. Treatment and symbols are as in Fig. 1.

While no change was observed at 26 days, total RNA contents were significantly augmented at 26 days (16.8%) and in the adults (3.9%) while its concentration increased only in the adults (10.5%).

Figures 3 and 4 show that pancreatic amylase content was strikingly increased at 26 (4%), 47 (43%), and 82 days (32%). Parallel increases were observed for chymotrypsin at all ages as well as for the soluble proteins except at 11 days. However, the amylase content was modified only in the adult. The concentration of amylase was increased only at 26 days (41%) while that of chymotrypsin showed an average augmentation of 50% at Days 26, 47, and 82. The soluble protein concentration was significantly modified by 17% at Days 26 and 82. On the contrary, lipase concentration remained unaffected at any time.

Discussion. These studies along with those previously published (5-7) confirm that the pancreas possesses growth capabilities that can be activated by the duodenal

hormone cholecystokinin-pancreozymin in adult rats. Our data also indicate that CCK-PZ can provoke hyperplasia in younger animals, those at the age of puberty (47 days).

However, when the treatment was given immediately after weaning (26 days) and on animals still being nursed by their mothers (11 days), hyperplasia following CCK-PZ was moderate. Indeed, the phenomenon is not clear cut since, in these animals, only one of the two criteria indicative of hyperplasia was positive. At 11 days, total DNA was not affected while DNA per gram body weight was significantly increased by 12.4%. In the 26-day-old rats, however, total DNA was elevated by 8.3% ($P < 0.05$) while DNA per gram body weight remained unchanged. This slight trophic effect of CCK-PZ observed at 11 and 26 days could be explained by the fact that at these ages, the pancreatic tissue is already in a state of intensive division as shown by Sesso *et al.* (14). Indeed, their studies indicate

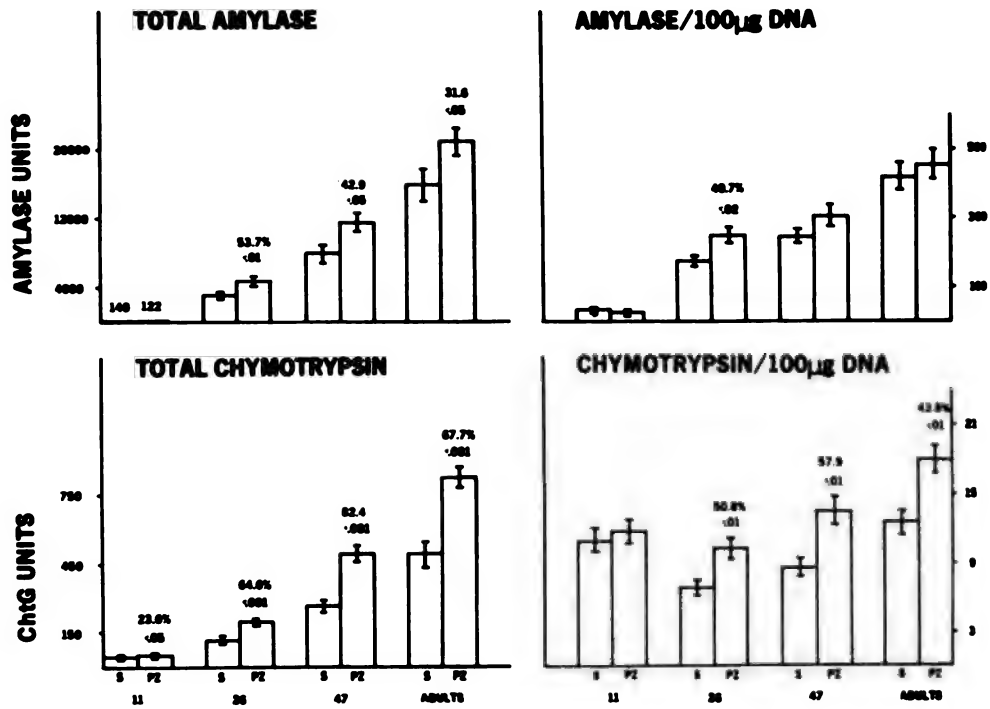


FIG. 3. Effects of chronic administration of cholecystokinin-pancreozymin on total pancreatic content and concentration of amylase and chymotrypsin. Treatment and symbols are as in Fig. 1.

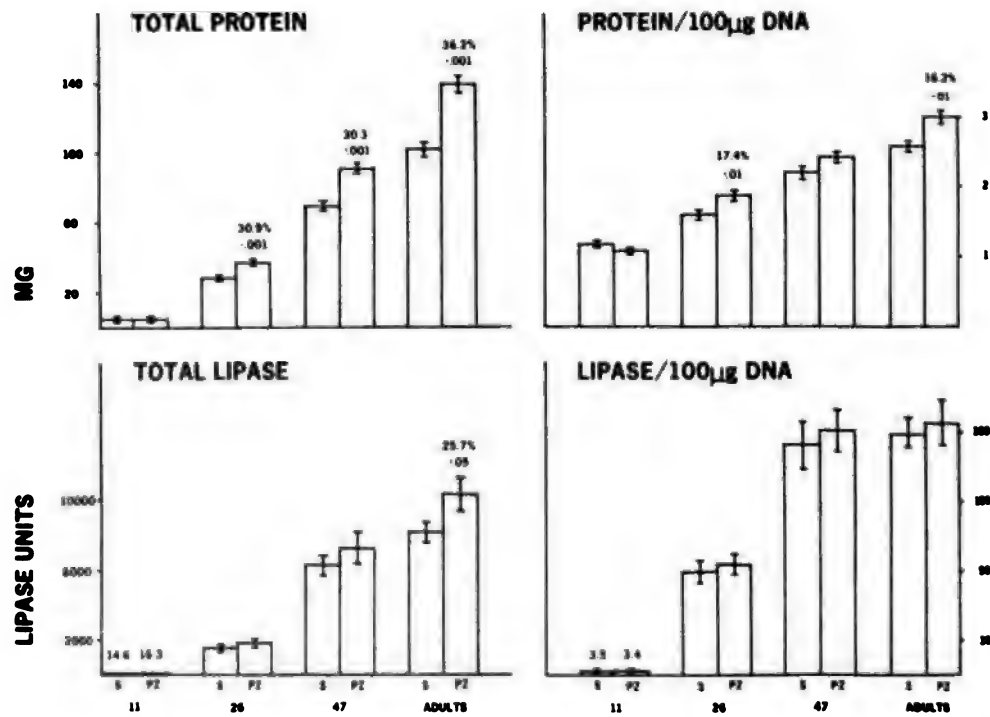


FIG. 4. Effects of chronic administration of cholecystokinin-pancreozymin on total pancreatic content and concentration of soluble protein (supernatant of 235,000g) and lipase. Treatment and symbols are as in Fig. 1.

that the mitotic indices are maximum between birth and 23 days; our data would thus suggest that the pancreatic tissue cannot maximally respond to trophic stimuli when the acinar cells are already in a high proliferative state.

Mainz *et al.* (6) show that CCK-PZ administration in adult rats was associated with both hypertrophy and hyperplasia. Their criteria for the evaluation of hypertrophy were increases in cell mass and in the concentration of RNA and proteins. In our studies, the cell mass (pancreatic weight/100 μ g DNA) was not significantly increased but the concentration of RNA (+10.5%, $P < 0.05$), soluble protein (+16.2%, $P < 0.01$), and chymotrypsin (+44%, $P < 0.01$) were observed while those of amylase and lipase remained at the control level. Barrowman and Mayston (7) also obtained augmented concentrations of RNA, proteins, and proteases. These observations have to be pointed out because they seem to indicate that CCK-PZ influences the synthesis of a specific class of digestive enzymes: those responsible for the hydrolysis of proteins. In the 47-day old rats, only the chymotrypsin concentration (58%, $P < 0.01$) was modified by CCK-PZ.

CCK-PZ treatment administered to weaning rats was associated with a well-defined hypertrophy since all the parameters (cell mass, RNA concentration, and that of all enzymes and proteins except lipase) were significantly increased over control level. However, 11-day-old rats did not respond to the hypertrophic effect of the hormone since all the parameters indicative of this effect were not changed. This lack of response from the gland does not indicate that it cannot hypertrophy at this stage of development since the pancreas of trypsin inhibitor-fed rats of this age has undergone both hypertrophy and hyperplasia (15).

These studies have presented the kinetics of the hyperplastic and hypertrophic effects

of chronic administrations of CCK-PZ. Hyperplasia is moderate in 11- and 26-day rats while well established in older animals (47 and 82 days). Hypertrophy cannot be detected in suckling rats (11 days) but is most evident at 26 days since the concentrations of all parameters studied, with the exception of lipase, are significantly increased. In the 47-day and adult rats, CCK-PZ provokes a selective augmentation in the concentration of RNA and chymotrypsin. Thus, we can say that the trophic effects of the duodenal hormone do exist but vary in intensity and specificity with the age of the animal.

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Crowding Stress and Adrenal Mitochondrial 11 β -Hydroxylation in Vitro^{1, 2} (39584)

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A variety of stressful environmental conditions are known to stimulate the activity of the hypothalamic-pituitary-adrenal complex. An increase in the population density of animals in natural as well as in laboratory conditions leads to adrenal hypertrophy, increased secretion of corticosteroids, as well as other responses associated with elevated release of ACTH (1-5). Slices of adrenal glands isolated from animals under situations of social or population density stress show enhanced corticosteroid release (2, 3). ACTH administration *in vivo* causes an increase in the capacity for 11 β -hydroxylation by adrenal mitochondria *in vitro* (6, 7). Physiological studies dealing with crowding stress are hampered by the complexities involving quantification of the stressor and adaptive responses of the adrenal in terms of blood corticosterone determinations (8). Since adrenal mitochondrial 11 β -hydroxylase activity can serve as an index of ACTH stimulation, measurement of this reaction *in vitro* offers a means of evaluating a stress response. The objective of the present investigation was to study the effects of various crowding densities in rats on the *in vitro* capacity of adrenal mitochondria to carry out the 11 β -hydroxylation reaction.

Materials and methods. Male Holtzman rats, initial body weight 150-170 g, were housed in wire mesh cages and fed on Purina laboratory chow and tap water *ad libitum*. The rats were maintained at 22-23° on a 14-hr light/10-hr dark cycle. The normal control rats were provided 110 cm² of floor space per rat. For crowding, the animals were housed at 50 or 30 cm² of floor space per rat. Animals were kept under these con-

ditions for 3 weeks before adrenals were obtained.

Since the hypothesis to be tested pertained to alterations in mitochondrial 11 β -hydroxylation in response to stress-associated elevation of endogenous ACTH, it was appropriate to validate the experimental procedure by employing hypophysectomized and ACTH-treated rats. Male rats were hypophysectomized through the intraaural approach using a Hoffman-Reiter stereotaxic instrument to position the animal. Adrenal glands were obtained from the hypophysectomized animals and from controls on Days 1, 5, and 10 following surgery. Exogenous ACTH (2 or 10 IU/rat; ACTHAR, Armour) was injected sc into intact male rats. Control animals were injected with isotonic saline. Adrenal glands were obtained 24 hr following injection. Both the removal of the adrenal glands and the subsequent incubation of mitochondria were carried out at approximately the same time of day.

Animals were decapitated, adrenal glands were removed and placed on filter paper moistened with 0.25 M sucrose in a cold chamber. In the hypophysectomized rats, the sella turcica area was examined following decapitation. In those cases where removal of the pituitary gland was considered to be incomplete, the adrenal glands were discarded. Glands were cleaned of adhering fat, weighed, and homogenized in 0.25 M sucrose containing 20 mM triethanolamine (TRA) buffer, pH 7.4. Mitochondria were obtained by the method of Peron and McCarthy (9). For each *in vitro* test adrenal mitochondria were prepared from 5 to 10 experimental rats and from a similar number of respective controls.

Aliquots of the mitochondrial fraction, suspended in the sucrose-TRA buffer mixture, were incubated in a media containing 20 mM TRA, pH 7.4, 50 mM nicotinamide, 180 nmole deoxycorticosterone (DOC),

¹ Supported in part by a grant (NGL-44-007-006) from NASA.

² Nomenclature of chemicals for which trivial names or abbreviations are used: deoxycorticosterone, DOC, 21-hydroxy-4-pregnene-3,20-dione; corticosterone, B, 11 β , 21-dihydroxy-4-pregnene-3,20-dione; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

either 10 mM isocitrate, succinate malate, or α -ketoglutarate as oxidizable substrate to support the reaction (9, 10). In all cases, 5 mM phosphate was included in the incubations containing 10 mM malate (10). The final volume of the incubate was brought to 2.0 ml with 0.154 M KCl. The reactions were carried out in a Dubnoff metabolic incubator at 37° under oxygen for 15 min and terminated by freezing the media. The corticosterone (B) content of the incubation media was analyzed using sulfuric acid fluorescence technique (12). Protein content was determined by the method of Lowry *et al* (13). The results are expressed as nanomoles B formed per milligram of mitochondrial protein in 15 min. In each case, data presented are based on separate measurements obtained from two or three different groups of animals.

Results. Adrenal mitochondria obtained from rats 5 or 10 days following hypophysectomy showed the expected reduction in capacity for 11β -hydroxylation of DOC *in vitro* (Fig. 1). The B production on Day 5 was more than 50% below the control value. On the first day following surgery, however, the corticosteroid conversion was consistently greater in the operated than in control rats.

Compared to controls, adrenal mitochondria obtained from rats 24 hr after ACTH injection showed an increased capacity to carry out 11β -hydroxylation in the presence of oxidizable intermediates (Fig. 2). Administration of 10 U of ACTH resulted in a two- to fourfold increase in B formation by mito-

chondria in the presence of succinate, α -ketoglutarate, or malate.

The data on the effects of crowding on substrate-supported mitochondrial 11β -hydroxylation are presented in Fig. 3. In groups with 50 cm² of floor space per rat, mitochondria showed little or no variation from the controls. A further reduction to 30 cm² of floor space per rat resulted in a marked increase in DOC conversion with each of the substrates tested.

In order to evaluate the duration of the mitochondrial response to crowding some rats housed at 30 cm² of floor space per rat for 3 weeks were returned to the control conditions, i.e., 110 cm² of floor space per rat. Following 3 weeks under the normal conditions the activity of the adrenal mitochondria were compared to those maintained for a period of 6 weeks under the control conditions. The capacity for *in vitro*

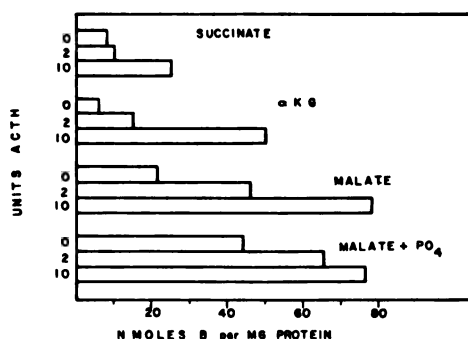


FIG. 2. *In vitro* hydroxylation of DOC by adrenal mitochondria prepared from intact male rats 24 hr after a single sc injection of ACTH.

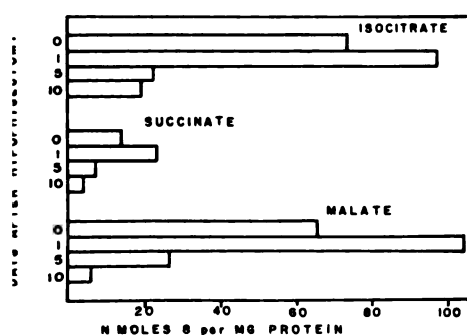


FIG. 1. The effect of time after hypophysectomy on *in vitro* hydroxylation of deoxycorticosterone by rat adrenal mitochondria. Values for Day 0 represent preparation from intact rats.

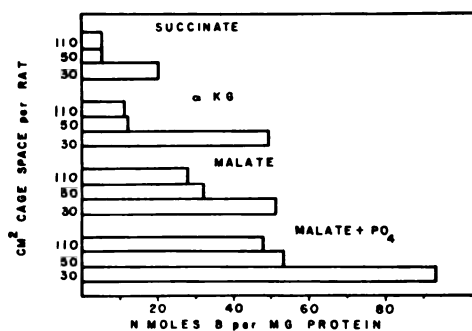


FIG. 3. *In vitro* hydroxylation of DOC by adrenal mitochondria prepared from intact male rats after housing for 3 weeks under the conditions indicated in the figure.

11β -hydroxylation in the crowded rats 3 weeks after transfer to control conditions remained elevated at levels similar to those obtained immediately at the termination of 3 weeks of crowding (as in Fig. 3). It appears that the effects of severe crowding result in the elevation of the capacity of adrenal mitochondrial for hydroxylation of DOC and the response persists for a period following the removal of the stressor.

Discussion. The results of this investigation demonstrate that the stressful severity of two different housing conditions can be differentiated on the basis of *in vitro* capacity of adrenal mitochondria to carry out the 11β -hydroxylation reaction. Studies of Christian and co-workers, as well as work by others (1-5), have amply demonstrated that crowding produces alterations in adrenal activity. Increases in the population density of previously individually housed mice resulted in an increase in adrenal weight, in the width of zona fasciculata and in *in vitro* corticosteroid production by the adrenal sections (see 1 and 2). These responses represent the generalized reaction of the organisms to stress. Within a population of a given density the potentially stressful stimuli may be due to the pressure of numbers as well as the member interactions (1). Whereas the effect of the former may be felt by all members of the population, the influence of the latter is, however, more selective. Social interactions between the members of a population are known to cause a greater degree of stress in the submissive than in the dominant members (1, 14). An intragroup variation in the degree of stressful reaction is, therefore, to be expected.

In the present study, the adrenals from the rats within the same cage were processed together. The measurements, therefore, represent the average of the cumulative stress response of a given group. Based on the group response, it is apparent that the decrease in housing space from 110 cm²/rat to 50 cm² did not result in any significant alteration in the hydroxylation response at the end of the 3-week period. A further reduction to 30 cm² of floor space per rat led to an elevation in the 11β -hydroxylation reaction, indicative of a stress response. It should be pointed out that in the group held at 50 cm²/rat, we cannot exclude the possi-

bility of an earlier or transient stress response followed by a normalization by the end of the 3-week period. If a transient response did occur in the 50-cm² groups, then the more dense housing conditions (30 cm²/rat) could be viewed as increasing the severity and/or duration of the stress response. It is of interest to note that in mice, a more aggressive species than the rat, increase in population to a minor extent causes elevation in stressful responses (1, 2, 4, 5).

The levels of 11β -hydroxylation obtained in the presence of oxidizable substrates (see Figs. 1-3) are comparable to those reported elsewhere (7, 9-11). The 11β -hydroxylation supported by an oxidizable substrate is related to its efficiency to generate intramitochondrial NADPH, with NADP⁺-linked oxidation of isocitrate being most effective in the rat. On the basis of the relative levels of DOC hydroxylated in the presence of various substrates it was possible to correlate the experimental regimens with alteration in 11β -hydroxylase activity.

The 11β -hydroxylase, an enzyme specific to adrenal mitochondria, catalyzes the final step in glucocorticoid formation, i.e., the conversion of DOC into B. ACTH exerts its trophic action in stimulating the first step in corticosteroidogenesis involving the conversion of cholesterol to pregnenolone. The rate of this reaction significantly increases within 10 min of ether stress (15). The 11β -hydroxylase activity also increases following ACTH (6, 7, 16) and decreases after hypophysectomy (17; Fig. 1). A single injection of ACTH elevates the conversion of DOC into B within 30 to 60 min and the effect can be seen after 24 hr (7; Fig. 2). It would thus appear that the elevation of 11β -hydroxylase activity, which is consistently noted on the first day following hypophysectomy (Fig. 1), is due to the ACTH release in response to the stress of etherization and surgery. However, the levels of 11β -hydroxylation 24 hr postsurgery tend to be higher than those 24 hr after administration of ACTH (Figs. 1 and 2). The nature of factors, released in addition to ACTH, responsible for this difference is uncertain.

The 11β -hydroxylase shows a peculiar time-associated activity after hypophysectomy (Fig. 1). After surgery this enzyme

activity remains at elevated levels at 24 hr. In contrast, it is well established that adrenal secretion decreases within minutes while adrenal sensitivity to exogenous ACTH diminishes within a few hours following hypophysectomy. The decrease in 11β -hydroxylation to less than 50% of the controls 5 days following surgery (Fig. 1) is consistent with the previously determined half-life of 4 days for this enzyme (17). The activity of the 11β -hydroxylase thus reflects levels of ACTH to which the adrenal gland had been previously exposed and likewise can be used as a measure of the severity of a stressor.

Relatively little is known concerning the physiological changes which persist following removal of a stressor. Our attempt to reverse crowding stress by returning the animals to the normal housing conditions did not result in a decline in 11β -hydroxylation. Since the half-life of the 11β -hydroxylase is 4 days (17), the high levels of DOC hydroxylation 3 weeks after the termination of crowding stress suggest the possible persistence of ACTH stimulation. The nature of the mechanism responsible for this response is obscure. It is known that prolonged administration of ACTH or chronic exposure to stress is associated with several pathological conditions which include cardiac hypertrophy, glomerulonephritis, and liver damage (1, 3, 18–21). It is conceivable that pathological conditions may themselves be associated with persistence of physiological changes in the post-stress period.

Quantitative ultrastructural studies have been undertaken on cardiac myocytes of rats in stress and in the post-stress period (McCarthy and Sohal, unpublished observations). Some cardiac changes, such as mitochondrial volume, lipid accumulation, and separation of intercalated disks, were more severe 3 weeks after removal from stress than immediately after crowding itself. Further studies are needed to elucidate the relationship between such changes and the persistence of apparent adrenal hyperactivity.

Summary. Adrenal mitochondria were obtained from rats subjected to two different crowding regimens in order to test the *in vitro* capacity for 11β -hydroxylation (11β -OH) as an index of stress. Compared to controls, 110 cm² of floor space per rat,

crowding at 30 cm²/rat markedly increased 11β -OH. Little change occurred when rats were held at 50 cm²/animal. High levels of 11β -OH persisted 3 weeks after the termination of severe crowding. This apparent failure to reverse crowding stress is viewed in terms of pathologic changes that appear to accompany crowding stress. On the basis of measuring 11β -OH capacity, it is possible to differentiate between the stressful effects of the housing conditions.

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Influence of Riboflavin Antagonists on Azo Dye Hepatoma Induction in the Rat¹ (39585)

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In 1936, Kinoshita (1) observed that feeding a diet containing 4-dimethylaminoazobenzene (DAB) caused the induction of hepatomas in rats. Kensler and co-workers (2) showed that excess riboflavin (Fig. 1, A) provided such experimental rats with protection against the carcinogenic action of the dye. Griffin and Baumann (3) and Miller and co-workers (4, 5) found that a decrease in riboflavin content of the liver occurred when DAB or 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) was added to the diet.

An azo reductase in the rat liver has been described (6, 7). The administration of DAB causes a decrease in the activity of this enzyme to cleave the carcinogen (8, 9) while the administration of excess riboflavin has been reported to enhance the activity of this enzyme (6, 8). However, evidence that this azo reductase is a flavoprotein has not been convincing.

More recently, Lambooy (10) has shown that when a critical balance has been struck between the quantities of 3'-Me-DAB and riboflavin administered, most of the rats (87%) do not develop hepatomas during 18 weeks, while some (13%) do. It was further shown that if in place of riboflavin, one or another of two vitamin-like homologs (Fig. 1, B and C) of riboflavin, both of which were equivalent to riboflavin in terms of growth, efficiency of food utilization, and survival (11), but both of which cause reduction of some enzymic activity (12, 13), was used, approximately 90% of the experimental animals developed hepatomas or precancerous conditions. Thus, restriction of the amount of exogenous riboflavin enhances while increased amounts inhibit hepatoma formation when DAB is adminis-

tered. Further, riboflavin analogs possessing less than complete vitamin-like replacement value for the vitamin, do not protect the rat when 3'-Me-DAB is administered.

There remains one more mechanism by which the role of riboflavin in the hepatoma induction process can be evaluated and that is to reduce the effectiveness of riboflavin through the simultaneous administration of potent antagonists of riboflavin. The most potent antagonists of riboflavin are 7,8-diethyl-flavin (Fig. 1, D) (14), a competitive antagonist of the vitamin, 7-chloro-8-methyl-flavin (Fig. 1, E) (15), a riboflavin analog possessing mixed vitamin-like and antagonist properties, and 7,8-diethyl-aminol-flavin (Fig. 1, F) (to be published in J. Med. Chem.), a riboflavin antagonist which, while still a flavin, possesses a side chain at position 10 other than D-ribityl. This report describes the effects that these antagonists have on the carcinogenic process induced by 3'-Me-DAB and how these findings provide additional support for the role of riboflavin in this process.

Materials and methods. Male rats of the Sprague-Dawley strain were used.² They were obtained from the supplier weighing 128-154 g; they were divided into five groups of 20 animals each and were housed two to a cage in quarters maintained under continuous temperature ($24 \pm 2^\circ$) and illumination (12 hr:12 hr) control. The rats were fed the following basic diet *ad libitum*. Vitamin B-complex free test diet³ was used, to each kilogram of which were added (milligrams): pteroylglutamic acid, 0.6; biotin, 1.5; thiamine-HCl, 20; pyridoxine-HCl, 20; riboflavin, 2; nicotinamide, 50; potas-

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² CFE rats from Carworth Division, Charles River Breeding Laboratories, Wilmington, Mass. 01887.

³ Sucrose 68%, casein 18%, corn oil 10%, salt mixture II 4%; obtained from Teklad Test Diets, Madison, Wis. 53711.

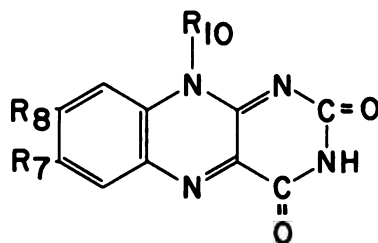


FIG. 1. Basic flavin structure.

R ₇	R ₈	R ₁₀	Trivial name
CH ₃ —	CH ₃ —	—CH ₂ —(CHOH) ₃ —CH ₂ OH	Riboflavin
C ₂ H ₅ —	CH ₃ —	—CH ₂ —(CHOH) ₃ —CH ₂ OH	7-Ethyl-8-methyl-flavin
CH ₃ —	C ₂ H ₅ —	—CH ₂ —(CHOH) ₃ —CH ₂ OH	7-Methyl-8-ethyl-flavin
C ₂ H ₅ —	C ₂ H ₅ —	—CH ₂ —(CHOH) ₃ —CH ₂ OH	7,8-Diethyl-flavin
Cl—	CH ₃ —	—CH ₂ —(CHOH) ₃ —CH ₂ OH	7-Chloro-8-methyl-flavin
C ₂ H ₅ —	C ₂ H ₅ —	—(CH ₂) ₂ —N(CH ₂ —CH ₂ OH) ₂	7,8-Diethyl-aminol-flavin

aminobenzoate, 50; calcium panto-60; inositol, 100; and (micro-yanocobalamin, 40. To the diet was added 1.5 g of choline chloride, and 20 liver oil containing 50 mg of menadione and 1 mmole (239 mg) of 3'-Methyl-Group A was fed the basic diet; Group B was fed the basic diet to which was added 10.2 mg/kg of 7,8-diethyl-flavin (Fig. 1, D); Group E-I was fed the basic diet to which was added 10.0 mg/kg of 7-methyl-flavin (Fig. 1, E); Group E-II was fed the same as Group E-I except that the amount of flavin was reduced to 5.0 mg/kg of diet; Group F was fed the basic diet to which was added 11.0 mg/kg of 7,8-diethyl-aminol-flavin (Fig. 1, F). The groups were fed their respective diets for a period of 18 weeks. At each time the animals were killed and livers were examined for hepatomas or other changes.

Results and discussion. Feeding a diet containing 2 mg of riboflavin and 1 mmole of 3'-DAB/kg of otherwise adequate diet to rats, protected the livers of the animals against hepatoma formation to the extent that 80% were found to be normal (Group A). These findings confirm earlier studies (10).

When animals (Group D) were fed the basic diet to which had been added the flavin antagonist flavin D, the benefit of the riboflavin was suppressed

as is evidenced by 80% of the animals developing hepatomas and 20% showing precancerous conditions. The antagonistic activity of the 7,8-diethyl-flavin is also illustrated by the significant reduction in the rate of growth of the animals receiving it.

When animals (Group E-I) were fed the above basic diet to which had been added flavin E, a flavin possessing both vitamin-like and antagonist activities, 30% of the animals died between the third and eighth week of the experiment. None of the animals that died possessed hepatomas at the time of death. As had been observed before (15), large quantities of this analog emphasized its antagonist properties. Only 25% of the livers of this group were found to be normal while 45% showed hepatomas (35%) or precancerous lesions (10%). When the immediately preceding experimental group was reconstituted but with the quantity of flavin E reduced to 5.0 mg/kg (Group E-II), all the animals survived, and furthermore, some of the vitamin-like properties of the analog were emphasized relative to the findings in Group E-I. For example, the gain in weight by this group was significantly greater than that of the control group (Group A). Only 11% of the animals in Group E-II developed hepatomas but the remaining 89% presented a gross appearance of livers not previously observed in our laboratories. The cirrhosis was severe but not unfamiliar, consisting of orange peel-like surface with deep segmentation and

quantities are equimolecular.

notching of the edges of all lobes. All lobes were "puffed" in appearance. The striking difference in appearance was the presence of very diffuse, very blanchd, whitish area of the livers. These areas were not the same as those listed as small white lesions in Table I [previously identified as precancerous (16)], and they were distributed among all lobes. The larger lobes, the median lobe, and the right and left lateral lobes in some cases showed half of their surfaces to be of this blanchd appearance. These areas were also observed in the livers of the two animals that had developed the tumors, but for rating purposes (Table I) the latter criterion was considered to be the more significant. These blanchd, whitish areas were not observed in any of the four other groups.

When animals (Group F) were fed the above basic diet to which had been added flavin F, the results were totally unexpected. This flavin had been shown to be an antagonist of riboflavin in riboflavin-deficient rats. After the first 2 weeks of the experiment, the rate of growth of the animals of this group strongly suggested that this analog was supplementing riboflavin and the rate of growth continued to be greater than that of Group A throughout the 18-week experimental period. When the animals of Group F were killed and examined the livers of all

were normal in appearance; there was no evidence of any abnormality.

Our extensive study of riboflavin analogs has firmly established that what is meant by "biological activity" of flavins is in need of considerable modification (see reference 14, pp. 358-362). In the case of the rat, we have shown that such biological activity might involve, to a considerable degree independently, growth, improved appearance, survival, reproduction, and inhibition of the beneficial influence of riboflavin. However, all of the above was based on observations made on flavins possessing the D-ribityl side chain at position 10. Our rationale (17) for the synthesis of flavins possessing the diethanolaminoethyl side chain at position 10, was to explore the possibility of developing a new class of antagonists of riboflavin. We had not anticipated that such an extreme departure from the normal side chain could be associated with vitamin-like activity. The particular details of the differences between two of the flavins used must be emphasized. Flavin D differs from the vitamin only in that it possesses ethyl groups in place of methyl groups at positions 7 and 8; it is clearly an antagonist of riboflavin in this system. Flavin F differs from the vitamin in that the ethyl groups have replaced the methyl groups at positions 7 and 8, but

TABLE I. INCIDENCE OF HEPATOMAS IN RATS FED RIBOFLAVIN, OR RIBOFLAVIN WITH VARIOUS RIBOFLAVIN ANALOGS, AND 3'-METHYL-4-DIMETHYLAMINOAZOBENZENE.

Flavin	Starting wt (g)	Wt gained (g) ^a	Liver findings				
			Normal ^b	Tumors ^c	Small lesions ^d	Cirrhosis ^e	Died
A	141 ± 2	342 ± 11	16/20 ^f (80)		4/20 (20)		
B	142 ± 1	289 ± 10 ^g		16/20 (80)	3/20 (15)	1/20 (5)	
E-I	143 ± 2	337 ± 18	5/20 (25)	7/20 (35)	1/20 (5)	1/20 (5)	6/20 (30)
E-II ^h	141 ± 2	430 ± 17 ⁱ		2/19 (11)		17/20 ^j (89)	
F	142 ± 1	394 ± 12 ^k	20/20 (100)				

^a Average weight gained by the surviving animals during the 18-week period ± SE.

^b Normal in terms of visual gross inspection.

^c Tumors varied from one to many; from 4 mm to 3 cm in diameter; these livers were cirrhotic.

^d Small lesions 1 to 3 mm in diameter, whitish in color, not yet grossly nodular, but clearly precancerous. These livers also showed cirrhosis.

^e Cirrhosis, but no small lesions nor tumors.

^f Number of animals showing change over the number of animals in the group. In parentheses is the percentage of the animals in the group showing change.

^g The *P* value (Student's *t* test) for the difference between this weight and that of the riboflavin group = 0.0008.

^h This group started 4 weeks after the other groups.

ⁱ The *P* value for the difference between this weight and that of the riboflavin group = 0.0001.

^j In addition to the severe cirrhosis, see text for description of appearance.

^k The *P* value for the difference between this weight and that of the riboflavin group = 0.0024. The difference between this weight and that of Group E-II was not considered significant (*P* = 0.100).

er, the side chain at position 10 is different from the normal D-ribityl. This study provides very convincing evidence that flavin F possesses vitamin-like properties in this test system. However, there are alternative possibilities which can be mentioned but not supported at this time.

If the azo reductase is important in protection against the induction of hepatomas by DABs, one might speculate that the flavin F stimulates its formation or activity, a role of which suggests vitamin-like properties, although only in a very limited and indirect sense. Flavin F might be destroyed essentially with the end result that the riboflavin is responsible for the apparent supplemental action of the analog. Another possibility is that flavin F may protect the liver against the effects of DAB by mechanisms unknown at present. All of the above mechanisms or possibilities are susceptible to study.

The influence of riboflavin on the induction of hepatomas in rats receiving DABs has now been demonstrated by all possible methods available.

Summary. The addition of a small amount of riboflavin to an otherwise adequate diet, a diet containing the hepatic carcinogen 2-thyl-4-dimethylaminoazobenzene, completely inhibits the carcinogenicity of the diet when fed to rats for 18 weeks. Most of the rats (80%) did not develop hepatomas. When the riboflavin antagonist 7,8-diethyl-7,8-dimethyl-7,8-dihydro-2-thyl-4-dimethylaminoazobenzene was also added to the above basic diet, all of the animals developed tumors or precancerous lesions (20%). 7-chloro-8-methyl-flavin was added to the above basic diet, which of its two properties, the vitamin-like or the riboflavin

antagonist properties, predominated depended on the quantity administered, but the flavin did not protect the livers from gross abnormalities. When the flavin 7,8-diethyl-aminol-flavin was added to the above basic diet, none of the animals developed hepatomas, suggesting that this flavin possesses strong vitamin-like properties or that it is capable of protecting the liver against the effects of the carcinogen.

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Enhanced Effect of Cyclophosphamide on Burkitt Lymphoma Cell Lines *in Vivo*¹ (39586)

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We have recently described a modified host-mediated assay system using human lymphoid cells cultured in diffusion chambers implanted into mice (1). Using this system and cytogenetic analysis as an indicator of the possible mutagenic effects of various agents, preliminary studies demonstrated that cyclophosphamide (CY) caused a significantly higher number of chromosome aberrations in the Burkitt lymphoma (BL) cell line B35M than in the normal-derived cell line SP-163. This result suggested that BL-derived cells were more sensitive to the chemotherapeutic drug CY than other lymphoid cell lines, and the present studies were initiated to investigate this possibility.

Reports on the cytostatic action of CY, an alkylating agent, or its ability to induce chromosome damage *in vitro* vary. Connors *et al.* (2) suggest that CY must undergo a metabolic conversion *in vivo* to yield the active antitumor and mutagenic agent, and the studies of Perry and Evans (3) using Chinese hamster cells (line CHO) support this notion. However, Nasjletti and Spencer (4) reported that CY could induce chromosome aberrations in cultures of human leukocytes, while Bishun (5) described similar effects in a BL cell line *in vitro*. Therefore, we have investigated the CY sensitivity of our cell lines *in vitro* as well as in diffusion chambers.

Materials and methods. Seven human lymphoid cell lines were used, three BL and four non-BL. The three BL cell lines were B35M, HR1K, and Raji (6), while the four non-BL cell lines were Wentzl (7), derived

from a patient with Down's syndrome, SP-163 (8), B411 (9), and RPMI-1788 (9). The latter three lines were all derived from the blood of normal individuals. The cells were cultured in medium RPMI-1640 supplemented with 10% fetal calf serum but without antibiotics.

The technique of culture in diffusion chambers (DC) has been described (1). The dry-sterilized DC were filled with 0.33 ml of a suspension containing approximately 5×10^5 cells. The filled DC were implanted into the peritoneal cavity of 6- to 8-week-old C3H/St mice under ether anesthesia. One day after DC implantation, the mice were injected ip with CY at a concentration of either 100 or 150 mg per kg body weight. Twenty-two hours after CY treatment, the animals received 0.5 ml of 0.04% colchicine ip, and the DC were removed 2 hr later, 24 hr after CY treatment. The DC were then treated with 0.5% Pronase in Hank's balanced salt solution containing 5% ficol for 60-90 min to disperse the cell aggregates. After removing the cells from the DC, the cell viability and number from each DC were determined. Cells were then treated with hypotonic solution and fixed in acetic acid:methanol (1:3). Flame-dried slides were made and stained with Giemsa.

The cytogenetic and cytostatic effects of CY *in vitro* were assessed in suspension cultures containing 10 ml of cells initially at 5×10^5 cells/ml. Cyclophosphamide at final concentrations of 1, 10, 50, and 100 $\mu\text{g/ml}$ was compared to control cultures. The cell viability counts and cytogenetic analyses were made 24, 48, and 72 hr after the addition of CY. Colcemid (0.04 $\mu\text{g/ml}$) was added 2 hr before collecting cells for chromosomal analyses. Cells with gaps, breaks, exchanges, dicentrics, and rings were recorded.

Results. With the exception of RPMI-1788, all the human lymphoid cell lines

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TABLE I. CHROMOSOME ABERRATIONS INDUCED BY CYCLOPHOSPHAMIDE IN BURKITT CELL LINES (B35M, HR1K, AND RAJI) AND IN NON-BURKITT LINES (SP-163, WENTZL, AND B411) CULTURED IN DIFFUSION CHAMBERS AND IMPLANTED INTO MICE.^a

Cell line	Dose (mg/kg)	No. of chambers	Total cells analyzed	Gaps (G)% (#G/cell)	Breaks (B)% (#B/cell)	Exchanges (E)% (#E/cell)	Dicentric (D)% (#D/cell)	Rings (R)% (#R/cell)	Multiple aberrations %	% Cells with aberrations
B35M	150	3	100	23.3 (0.37)	49.6 (1.20)	20.4 (0.25)	4.1 (0.04)	-	7.9	70.0
	0	4	180	3.8 (0.04)	3.2 (0.03)	-	-	-	-	3.9
HR1K	150	3	85	24.7 (0.54)	42.4 (1.04)	10.6 (0.18)	3.5 (0.06)	1.2 (0.01)	9.4	57.6
	0	3	80	3.8 (0.05)	2.5 (0.05)	-	-	-	-	5.0
Raji	150	4	55 ^b	29.1 (0.47)	43.6 (1.31)	14.5 (0.27)	5.5 (0.05)	-	14.5	70.9
	100	1	11 ^b	27.3 (0.45)	45.5 (1.09)	9.1 (0.18)	9.1 (0.09)	-	18.2	81.8
	0	2	60	5.0 (0.07)	3.3 (0.03)	1.7 (0.02)	-	-	-	5.0
SP-163	150	5	136	6.0 (0.06)	15.5 (0.25)	2.5 (0.03)	1.5 (0.02)	0.5 (0.01)	-	22.6
	0	6	150	4.0 (0.04)	5.0 (0.05)	-	2.0 (0.02)	-	-	6.0
Wentzl	150	4	100	5.0 (0.06)	6.0 (0.09)	5.0 (0.05)	3.0 (0.04)	-	-	15.0
	0	3	100	3.0 (0.04)	3.0 (0.03)	1.0 (0.02)	-	-	-	6.0
B411	150	2	30 ^b	6.7 (0.07)	6.7 (0.10)	3.3 (0.03)	3.3 (0.03)	-	3.3	20.0
	100	3	100	5.0 (0.07)	6.0 (0.08)	2.0 (0.02)	-	-	-	14.0
	0	4	100	3.0 (0.05)	5.0 (0.05)	2.0 (0.02)	-	-	-	5.0

^a Breaks include chromatid or chromosome breaks and fragments. Cells with exchange, dicentric, or ring may have breaks which were also included in the cells with breaks. Cells having more than eight aberrations (multiply aberrations) were not recorded in the other categories.

^b Mitoses were suppressed; only a few metaphases could be scored.

grew well in DC implanted in mice. Line RPMI-1788 was, therefore, not included in the *in vivo* cytogenetic studies. Cell lines B35M, HR1K, and SP-163 proliferated more rapidly in DC than lines Raji, Wentzl, and B411. The former three lines increased three to four times the initial concentration in 2 days while the latter three lines approached one doubling. Cyclophosphamide reduced the growth rate of all the cell lines.

The effects of CY on the chromosomes of cells from the six cell lines cultured in DC are shown in Table I. It is evident that treatment with CY caused a high incidence of chromosomal aberrations in all six lines, but that the BL cell lines were uniformly more sensitive to CY than the non-BL cell lines. The incidence of aberrations in the BL cell lines ranged from 58–82% while this was only 14–23% in the non-BL lines. This difference in sensitivity between the two groups of cell lines was highly significant ($P < 0.001$).

Studies carried out in tissue culture were uniformly negative. All the cell lines grew well, including RPMI-1788, and CY in various concentrations from 1–100 $\mu\text{g/ml}$ did not affect their growth rates. Cytogenetic analysis of 50 metaphases from control flasks and flasks treated with various durations and concentrations of CY in all the cell lines revealed no effects of CY on chromosomes.

Discussion. Our results have shown that BL cells carried in mice in DC were significantly more sensitive to CY than non-BL cells. The reasons for such a high sensitivity are presently under investigation. The *in vitro* studies would suggest that the BL cells do not activate CY directly, although the presence of an inhibitor of this activation in the tissue culture medium has not been ruled out. It is possible that the BL cells stimulate the host's activation of CY and result in a higher concentration of the active metabolite in the peritoneal fluid, a possibility which can be tested using the peritoneal fluid directly. It has been reported (10) that certain cancer cells are more sensitive to CY *in vivo* than normal cells, and, thus, the enhanced sensitivity observed in the present work may reflect an intrinsic property of the

BL cell unrelated to activation phenomena. For example, the BL cells used in the present studies are all hyperdiploid. Zech *et al.* (11) recently reported that cells from many BL and BL biopsies had a marker chromosome, 14q+. The non-BL cell lines SP-163 and B411 are diploid while the Wentzl line retains the characteristic constitution of Down's syndrome. Our studies, therefore, open several avenues for future investigation while demonstrating further specific effects of cyclophosphamide *in vivo* but not *in vitro*.

Summary. Cells from three Burkitt lymphoma lines and three normal-derived human lymphoid lines were cultured in diffusion chambers implanted into mice. Cytogenetic analysis was made on all these cell lines following treatment of the animals with cyclophosphamide. The Burkitt lymphoma cell lines were uniformly more sensitive to cyclophosphamide than the normal-derived cell lines, having a chromosome aberration incidence of 58–82% compared to 14–23% for the latter cell lines. Studies in tissue culture failed to reveal either the cytostatic effect of cyclophosphamide or an effect on chromosomes.

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Triamcinolone Activation of Renal Ammonia Production (39587)

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onic metabolic acidosis leads to activation of the mitochondrial glutaminase pathway (1, 2) resulting in a marked increase in renal ammonia production. This static response to acidosis appears to be mediated by the adrenal glands since plasma corticosterone levels are elevated in response to acid loading (3) while activation of the mitochondrial pathway does not occur in adrenalectomized animals (3, 4). Frequently, corticosteroids appear to play a key role in the adaptive increase in ammonia production. If this is so, administering exogenous glucocorticoids can elicit a renal response, via activation of the mitochondrial pathway, which resembles the response seen during acid loading. This report demonstrates that triamcinolone activates the mitochondrial pathway and results in a pattern of ammonium excretion similar to that observed in acidosis.

Materials and methods. Twenty-four male Sprague-Dawley rats weighing between 350 and 500 g were divided into two groups. One group was studied *in vivo* while the other group provided kidneys for studies of ammonia production by the isolated perfused kidneys.

In vivo study. Twelve rats were housed individually in a metabolic cage for the collection of 24-hr urine samples, and maintained on Purina rat chow and tap water *ad libitum*. After 3 control days, six of the rats were given triamcinolone (Sigma), 1 mg/kg/day in two divided doses by intramuscular injection of the hormone suspended in 0.5 ml of sodium chloride; six other rats received 0.5 ml of 0.9% sodium chloride. Following 3 days of treatment, the injections were suspended and 24-hr urine samples were collected for a subsequent 2-day period. Daily urine samples were collected, stored, and analyzed as already described (5). Urinary and plasma sodium and

potassium concentrations were determined by flame photometry; plasma total CO₂ was measured manometrically on a Natelson microgasometer (6) while urine pH was monitored on an Orion pH meter.

In vitro study. Six rats were administered triamcinolone, 1 mg/kg for a 24-hr period prior to the isolation and perfusion of their kidneys (2, 7); six others were sham-treated and employed as controls. The kidneys were perfused with an artificial plasma solution (7) and dextran T-110 (Pharmacia) was saturated with 95% O₂:5% CO₂ and adjusted to pH 7.4 Carrier L-glutamine at an initial concentration of 1 mM and tracer amounts of L-[μ-¹⁴C]glutamine (specific activity 127 μCi/mole, New England Nuclear) were the sole exogenous substrates. The perfusion circuit, normally opened to the atmosphere, was converted to a closed system by the modification introduced by Trimble and Bowman (8). Kidneys were perfused for 60 min after which the PO₂ of the perfusion media, measured by a Gilson oxygraph, was always in excess of 200 mm Hg. Concentrations of ammonia, glutamine, glucose, and radioactive CO₂ were measured at 15-min intervals; changes in substrate uptake, glutamine, product formation ammonia, ¹⁴CO₂, and glucose were linear over the perfusion period.

Analysis. Ammonia and glutamine were measured as previously published (2, 9); glucose was determined enzymatically using a modification of the glucose oxidase method (10). Radioactive CO₂ was liberated with 6 N HCl and completely trapped in hyamine hydroxide in the center well of a microdiffusion chamber after 90 min. All analyses were performed in triplicate. Calculations of the rates of glutamine uptake and ammonia and glucose production have been described (2, 11). The conversion of L-glutamine to CO₂ was calculated from the

appearance of $^{14}\text{CO}_2$ as formulated below. increase their excretion levels four- to five-
 $\mu\text{mole L-glutamine}, t = 0 \times \text{cpm } ^{14}\text{CO}_2, t = 60/$

$$\text{cpm}, t = 0 \text{ L-}[^{14}\text{C}]\text{glutamine} = \mu\text{mole glutamine to CO}_2$$

Statistics. All data were analyzed using the Student's *t* test.

Results. Figure 1 presents the influence of a single injection of triamcinolone upon 24-hr ammonium excretion measured at 4-hr intervals. Excretion level increased 365% during the first 4-hr period, peaked at 8 hr, and thereafter declined. Cumulatively, excretion rate was $1329 \mu\text{mole } 24 \text{ hr}^{-1}$ with the hormone compared to $628 \mu\text{mole } 24 \text{ hr}^{-1}$ for control giving only a twofold overall increase. Consequently, triamcinolone was administered at 12-hr intervals, $1 \text{ mg kg}^{-1} 24 \text{ hr}^{-1}$. As a result (Fig. 2), 24-hr ammonium excretion rose to greater than threefold during the first day, to fourfold after the second day, and to greater than fourfold on the third day. With cessation of triamcinolone, excretion levels promptly dropped to pretreatment values after 48 hr. In comparison, rats receiving ammonium chloride

fold over a several day period (12, 13); withdrawing the acid load causes a sharp fall off in the rat with ammonium excretion returning to control values after 2 days (13, 14). Clearly, triamcinolone can induce a pattern of ammonium excretion similar to that observed with acid loading despite the absence of an acid stimulus.

The effect of triamcinolone upon ammonium excretion is apparently not dependent upon potassium depletion since urinary potassium levels are not significantly elevated nor does hypokalemia occur (Table I). Nor is ammonium excretion secondary to an augmentation in a putative sodium for hydrogen exchange since urinary sodium increases while hydrogen ion excretion tends to decrease. As a result of triamcinolone's stimulation of ammonia production excess, acid is eliminated in the form of ammonium causing metabolic alkalosis, plasma total

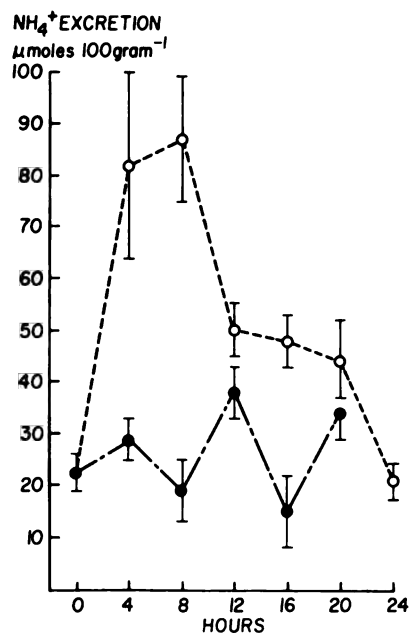


FIG. 1. Influence of a single daily injection of triamcinolone 1 mg kg^{-1} , upon ammonium excretion collected at 4-hr intervals. Points are means \pm SE from six sham (closed circles) and six hormone-injected, (open circles) rats.

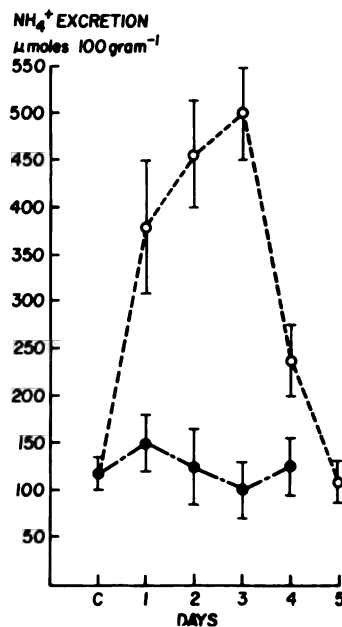


FIG. 2. Influence of triamcinolone, 1 mg kg^{-1} , administered as a divided dose at 12-hr intervals on 24-hr ammonium excretion over a 3-day period. Results are means \pm SE from six rats in control (closed circles) and six hormone-injected (open circles) rats.

TABLE I. INFLUENCE OF TRIAMCINOLONE UPON URINE AND PLASMA ELECTROLYTES.

Day	Urine				Plasma	
	Volume (ml)	pH (units)	Na ⁺ (mEq)	K ⁺ (mEq)	HCO ₃ (mEq)	K ⁺ (mEq)
Control	17 ^b	6.81	1.48	2.83	25.8	4.2
(6) ^a	±3	±0.10	±0.33	±0.73	±2.3	±0.5
1 ^c	32 ^d	6.94	3.18 ^d	3.56	33.4 ^d	5.6 ^d
(6)	±4	±0.07	±0.72	±0.56	±3.6	±0.7
2 ^c	30 ^d	6.94 ^d	1.96	2.28	32.8 ^d	4.5
(6)	±6	±0.05	±0.42	±0.12	±1.9	±0.4
3 ^c	21	7.04 ^d	1.25	2.59	36.5 ^d	4.1
(6)	±5	±0.11	±0.25	±0.22	±4.1	±0.6

number of rats.

mean ± SE.

triamcinolone 1 mg kg⁻¹ per day.

significantly different from control ($P < 0.05$).

33.4 mM. The high level of ammonia excretion is therefore all the more extraordinary in view of the limitations placed on ammonia excretion by the systemic pH and near neutral urine pH.

Ammonia production by isolated renal kidneys from rats administered triamcinolone for 24 hr is shown in Table II. Ammonia production increased 348% ($P < 0.01$) and glutamine uptake 232% ($P < 0.01$), while the rise in the ammonia proper glutamine uptake ratio, 1.32 to 1.33, indicates a shift in glutamine utilization from predominant γ -glutamyl-transferase to the more efficient mitochondrial glutamate dehydrogenase pathway (5, 16). Consistent with shift to the mitochondrial pathway is the increase in the glucose and ¹⁴CO₂ production, the end products of glutamine utilization via the mitochondrial pathway. Glucose production rose from 2.1 ± 0.4 to 8.9 ± 1.6 hr⁻¹ ($P < 0.01$), while the micro-amount of glutamine converted to CO₂ increased from 3.0 ± 0.8 to 11.1 ± 2.5 μ mole hr⁻¹ ($P < 0.01$).

Discussion. Ammonium excretion is markedly elevated by triamcinolone injection (2) as the result of activation of the renal glutaminase I pathway (Table I). This stimulation of renal ammonia production is not secondary to tubular ion excretion or to potassium depletion (Table I). The activation occurs promptly (Fig. 1), being consonant with an effect of triamcinolone upon the inner mitochondrial membrane's permeability to glutamine. An

TABLE II. EFFECT OF TRIAMCINOLONE ON AMMONIA PRODUCTION AND PATHWAYS OF GLUTAMINE (Gln) UTILIZATION.

	NH ₃ production	Gln uptake	Glucose production	¹⁴ CO ₂ ^a
Control	22.9 ^b	17.4	2.1	3.0
(6)	±1.7	±0.6	±0.4	±0.8
Triamcinolone	79.8 ^d	40.5 ^d	8.9 ^d	11.1 ^d
(6) ^c	±3.0	±1.4	±1.6	±2.5

^a Micromoles glutamine converted to ¹⁴CO₂ per hour.

^b Mean ± SE.

^c Triamcinolone administered 1 mg kg⁻¹ to six intact rats and their kidneys perfused as described in Methods.

^d Significantly different from control kidney ($P < 0.01$).

increase in substrate glutamine availability within the inner compartment could adequately explain both the renal response to exogenous glucocorticoids as well as the acidosis.

Endogenous glucocorticoids appear to play the key role in the normal response to acidosis since acid loading elevates systemic corticosterone levels (3), while in the absence of the adrenals acidosis does not activate the mitochondrial pathway (3, 4). In fact, pH changes appear to have relatively minor, if any, direct effect to enhance ammonia production (13). Consequently, potassium depletion was suggested as an intermediary factor in ammonia production activation. Yet, the adaptive increase in ammonia production occurs despite maintenance of potassium levels (12) while glucocorticoids can stimulate ammonia production

(Table II) causing metabolic alkalosis in the absence of alterations in potassium homeostasis (17).

Summary. Administering triamcinolone, $1 \text{ mg kg}^{-1} 24 \text{ h}^{-1}$, results in increased ammonium excretion which resembles that observed during acid loading. This increase cannot be attributed to tubular ion exchanges or potassium depletion but, rather, is due to triamcinolone's effect upon renal ammonia production. The three- to fourfold increase in ammonia, glucose, and $^{14}\text{CO}_2$ production is consistent with glucocorticoid mediation of the mitochondrial glutaminase I activation.

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tion of Cytomegalovirus Antibody by Immune Adherence Hemagglutination (39588)

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odies to cytomegalovirus (CMV) s can be detected by a variety of ues including complement fixation -3), neutralization (4), indirect he- tination (5, 6) immunofluorescence id platelet agglutination (8). Al-

heterogeneity exists among CMV s from different sources, the rela- road reactivity and commercial avail- of CMV antigens for CF as well as performance have made CF the most ly used serologic technique to detect y to CMV (anti-CMV). Recently detect antigens and antibodies asso- with hepatitis A and B viruses have rformed by immune adherence he- tination (IAHA), a complement-me- ssay more sensitive than CF (9-14). sent report describes the application A to the detection of anti-CMV in multiply transfused patients.

ials and methods. CMV antigen. antigen of the AD 169 strain was ed from Flow Laboratories (Rock- d). The antigen had been obtained ption of infected WI38 cells. Prior was stored at -70°. Control antigen :pared from disrupted, uninfected ells.

samples. A pool of human serum o have a CF titer of 1:32 was used as ve anti-CMV control. For compari- CF and IAHA, serum samples were d from 36 multiply transfused pa- ho had undergone open heart sur- 5). All samples were heated at 56° nin to inactivate complement.

rs. Veronal-buffered saline (VBS)

(pH 7.5) prepared as described by Kabat and Mayer (16) and supplemented with 0.1% bovine serum albumin (BSA) was used as diluent for antigen, antibody, and complement. A 40-mM EDTA solution in VBS-BSA was prepared as the diluent for human red blood cells and dithiothreitol (DTT).

Guinea pig complement. Guinea pig complement was obtained from a commercial source (Texas Biological Laboratories, Inc., Fort Worth, Texas) and diluted 1:75 for IAHA. Complement was prepared fresh in BSA-VBS diluent immediately prior to use.

Human red blood cells for IAHA. Type O, Rh-positive human red blood cells were obtained from normal volunteer blood donors. Because there is marked variation in red blood cell sensitivity as the indicator cell, red cells with the greatest sensitivity in IAHA were selected as the indicator cells in all subsequent tests. After collection, the red cells were suspended in 4 vol of Al-sever's solution and preserved this way for 3 to 4 weeks at 4°. Beyond this period, the cells became progressively less sensitive as indicator erythrocytes. Prior to use, the red cells were washed once with EDTA-VBS-BSA buffer, then diluted in the same buffer to yield a 1% (vol/vol) suspension.

IAHA test to detect anti-CMV. Optimum concentrations of CMV antigen and anti- body were determined by checkerboard ti- tration of antigen against reference antise- rum. In subsequent tests with the same lot of CMV antigen, the antigen was diluted in VBS-BSA to yield 4 IAHA units in 25 μ l.

Duplicate sets of serial twofold dilutions were prepared in polystyrene U-bottom mi- crotiter plates (Linbro Chemical Co., New Haven, Conn.) with 25- μ l microdiluters (Cooke Engineering Co., Alexandria, Va.). Twenty-five microliters of diluted antigen was added to one set of serum dilutions and

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VBS-BSA buffer was added to the second. The plate was shaken briefly on a microtiter micromixer (Cooke Engineering Co.) and incubated in a humid chamber overnight at 4°. The following morning, 25 μ l of freshly diluted guinea pig complement was added to each well, and the plate was briefly shaken and incubated for 40 min at 37°. Without delay, 25 μ l of DTT (3 mg/ml) was added to each well followed by 25 μ l of human red blood cell suspension. After thorough agitation on a micromixer, the plates were incubated at room temperature until negative hemagglutination patterns appeared in control wells, approximately 1 hr later. Hemagglutination was evaluated on a scale of zero to four, and patterns of three or four were considered positive, provided nonspecific hemagglutination was not observed in the corresponding control (buffer only) well. Immune adherence hemagglutination titers of anti-CMV were expressed as reciprocals of the highest serum dilution at which hemagglutination was observed.

A similar method was employed to determine IAHA titers of CMV antigen. Four IAHA units of anti-CMV serum in 25 μ l of VBS-BSA was added to wells containing serial twofold dilutions of antigen.

CF test to detect anti-CMV. The same microtiter equipment and guinea pig complement were used to determine anti-CMV and CMV antigen titers by CF. For each well, 2 units of hemolysin and 1.8 to 2.0 units of complement were added according to the microtiter CF procedure described by Sever (17). Complement fixation titers of anti-CMV were expressed as reciprocals of the highest dilution at which 25% (3+) or less (4+) hemolysis was observed.

Results. Relative sensitivity. The relative sensitivity of IAHA and CF for the detection of CMV antigen and anti-CMV was determined in a checkerboard titration of a reference antiserum against CMV antigen (Fig. 1). For the detection of antigen, IAHA was 4- to 16-fold more sensitive than CF; IAHA was two- to eightfold more sensitive in the determination of anti-CMV. On the basis of this titration, CMV antigen was used at a dilution of 1:32 for IAHA and 1:8 for CF in subsequent assays of anti-CMV. At these dilutions there were 4 IAHA or CF units in 25 μ l of antigen reagent. Neither

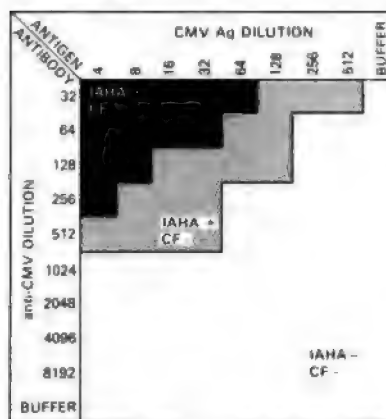


Fig. 1. Checkerboard titration of cytomegalovirus antigen (CMV Ag) against antibody to CMV (anti-CMV) by immune adherence hemagglutination (IAHA) and complement fixation (CF).

CF nor IAHA reactivity was observed when control antigen was used.

Anti-CMV in sera of transfused patients. Paired serum samples collected at least 1 month apart from multiply transfused patients were tested for anti-CMV by CF and IAHA (Table I). Sixteen of these 36 patients were selected on the basis of a sero-response to CMV antigen as determined by CF in previous studies. Of 16 patients with at least fourfold anti-CMV rises measured by CF, 14 demonstrated at least four-fold rises by IAHA. Two patients (Numbers 15 and 16) with minimal rises in anti-CMV measured by CF showed no response by IAHA; both had preexisting antibody and developed no more than a two-fold convalescent antibody rise in the IAHA test. Although proportional increases of anti-CMV titer between pretransfusion and late samples were comparable between CF and IAHA, pretransfusion titers were slightly higher and convalescent titers were significantly higher when measured by IAHA for the 14 patients with anti-CMV rises by both techniques (Fig. 2). Sixteen patients (Numbers 21 through 36) who showed no rise in anti-CMV by CF showed no change in titer by IAHA; however, four patients (Numbers 17-20) had measurable rises by IAHA but no antibody response detected by CF. Seven patients had no detectable anti-CMV by CF in either sample but had detectable stable titers of anti-CMV by IAHA in both (Num-

TABLE I. ANTI-CMV^a IN PAIRED SERA OF MULTIPLY TRANSFUSED PATIENTS.

Patient	Date ^b	Anti-CMV inverse titer		Patient	Date ^b	Anti-CMV inverse titer	
		CF ^c	IAHA ^d			CF ^c	IAHA ^d
1	4 Weeks	2	64	19	Pre	64	256
	8 Weeks	128	2048		22 Weeks	<256 ^e	1024
2	Pre	8	<8	20	Pre	16	256
	9 Weeks	64	256		11 Weeks	<64 ^e	≥2048
3	Pre	32	256	21	Pre	16	128
	14 Weeks	128	1024		8 Months	32	128
4	Pre	4	8	22	Pre	32	64
	24 Weeks	64	2048		24 Weeks	32	64
5	4 Weeks	<2	<8	23	4 Weeks	32	64
	11 Months	512	4096		12 Weeks	16	64
6	Pre	32	256	24	Pre	32	128
	23 Weeks	128	1024		10 Weeks	<128 ^e	128
7	Pre	<2	<8	25	Pre	<2	<8
	19 Weeks	32	128		8 Months	<8	<8
8	Pre	<2	<8	26	Pre	<4	<8
	17 Weeks	32	32		7 Months	<8	<8
9	Pre	<2	8	27	Pre	64	512
	22 Weeks	16	32		26 Weeks	<256 ^e	1024
10	Pre	<2	16	28	Pre	8	64
	10 Weeks	64	512		25 Weeks	<32 ^e	64
11	2 Weeks	<2	32	29	Pre	8	128
	13 Weeks	64	256		8 Months	<32 ^e	64
12	Pre	<2	<8	30	2 Weeks	<2	8
	10 Weeks	64	256		12 Weeks	<2	16
13	Pre	<2	8	31	Pre	<2	32
	20 Weeks	64	128		24 Weeks	<8	32
14	4 Weeks	<2	32	32	4 Weeks	<8	256
	10 Weeks	128	512		10 Months	<8	512
15	Pre	4	64	33	4 Weeks	<16	32
	24 Weeks	32	128		7 Months	<16	32
16	Pre	<2	8	34	Pre	<4	128
	17 Weeks	32	16		26 Weeks	<4	64
17	Pre	<2	<8	35	Pre	<2	64
	24 Weeks	<8	64		26 Weeks	<8	64
18	Pre	<2	<8	36	10 Weeks	<8	512
	23 Weeks	<8	512		12 Months	<8	256

^a Antibody to cytomegalovirus antigen.^b Relative to transfusion.^c Complement fixation.^d Immune adherence hemagglutination.^e These sera were tested beginning at a dilution fourfold greater than the titer of the pre-transfusion sample.

ers 30–36). The Kendal coefficient of association (18) between anti-CMV rises detected by CF and IAHA was 0.671 ($P < 0.001$) (Table II). In tests of reproducibility, no two determinations varied by more than one dilution when IAHA testing was repeated on the same serum sample.

Kinetics of anti-CMV development. The temporal relationship between the development of CF and IAHA antibody to CMV antigen was evaluated in serial serum samples obtained from two of the individuals evaluated above. Patient No. 4 had minimal pretransfusion anti-CMV, which remained

unchanged for 7 weeks as determined by CF. Anti-CMV detected by IAHA began to increase 2 weeks after transfusion. After 7 weeks, CF and IAHA antibody titers rose in parallel fashion, but IAHA titers remained higher than CF titers (Fig. 3a). Pretransfusion anti-CMV was not detected by either test in serum from patient No. 5. Between 6 and 16 weeks after transfusion, anti-CMV developed and was detected by both IAHA and CF. For each sample, the IAHA titer was slightly higher than the CF titer, but the patterns of changing titer were similar (Fig. 3b).

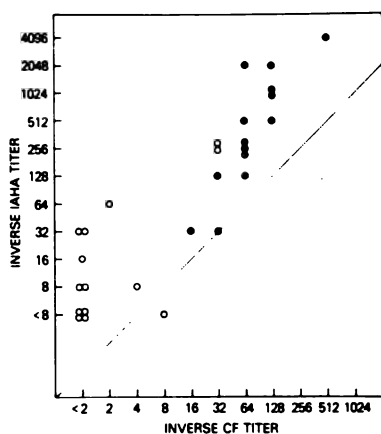


FIG. 2. Immune adherence hemagglutination (IAHA) titers expressed as reciprocal of dilution (given as inverse titers) plotted as a function of complement fixation (CF) titers of pretransfusion sera (○) and post-transfusion sera (●) for 14 patients with rises in titer of antibody to cytomegalovirus. Points falling above the diagonal line represent enhanced sensitivity of IAHA compared to CF.

Discussion. In immune adherence hemagglutination, there is complement-mediated agglutination of indicator erythrocytes rather than the hemolysis observed in complement fixation tests. Surface receptor sites on nonsensitized red blood cells adhere to the third component of complement (C_3) which is bound to an antigen-antibody complex and complement components C_1 , C_4 , and C_2 . Dithiothreitol is added to protect C_3 from C_3 -inactivator and, thus, to stabilize the hemagglutination pattern (9). The term "immune adherence" was coined by Nelson (19) to describe a phenomenon recognized half a century earlier (20, 21). To Nishioka (22) goes the credit for characterizing complement component participation in immune adherence and popularizing IAHA as a sensitive technique for the detection of bacterial and viral antigens. Subsequent work by Ito and Tagaya (23) established the usefulness of IAHA for detecting a variety of viral antigens and antibodies and demonstrated a 10- to 100-fold increase in sensitivity over complement fixation tests. Since 1971, IAHA has been used extensively in Japan to detect hepatitis B surface antigen and antibody. With sensitivity 40 to 100 times greater than the complement fixation test and comparable to radioimmunoassay

techniques, IAHA is used routinely to screen blood donors and for epidemiologic investigations (9, 10). Most recently, this sensitive assay has been used to detect antigens and antibodies associated with hepatitis A virus (11, 12, 14) and hepatitis B core antigen and antibody (13).

Cytomegalovirus antigen used in this study was obtained from the supernatant of pelleted, disrupted cells in which CMV was grown. Despite the lack of additional purification, the antigen preparation was relatively clean and easily usable in the test. Still, several discrepancies occurred between IAHA and CF results. In two cases of fourfold titer increases detected by CF, only twofold rises were detected by IAHA. For these two, pretransfusion antibody, undetected by CF, was observed by IAHA. In addition, cases 30-36 had no detectable anti-CMV by CF, but anti-CMV was demonstrated by IAHA in both specimens, and IAHA detected four significant serologic responses which were missed by CF. A factor which further compounds the difficulty of assessing these serologic events is the heterogeneity of CMV antigens and the incomplete cross-reactivity between them (24). Some of the discrepancies noted in this study may result from differing degrees of cross-reactivity among CMV strains as measured by CF and IAHA, since only the AD 169 strain was employed as antigen in this study. In general, however, the parallelism between CF and IAHA in all cases of convincing sero-response and the enhanced sensitivity of IAHA make IAHA an attractive alternative to CF. Moreover, the increased sensitivity of IAHA reveals that a greater proportion of anti-CMV responses are reinfection rather than primary exposures. Although other CMV strains were not available for testing, their inclusion in

TABLE II. CONCORDANCE BETWEEN RISES IN ANTI-CMV^a TITERS DETECTED BY CF^b AND IAHA.^c

CF	IAHA	
	Rise	No rise
Rise	14	2
No rise	4	16

^a Antibody to cytomegalovirus.

^b Complement fixation.

^c Immune adherence hemagglutination.

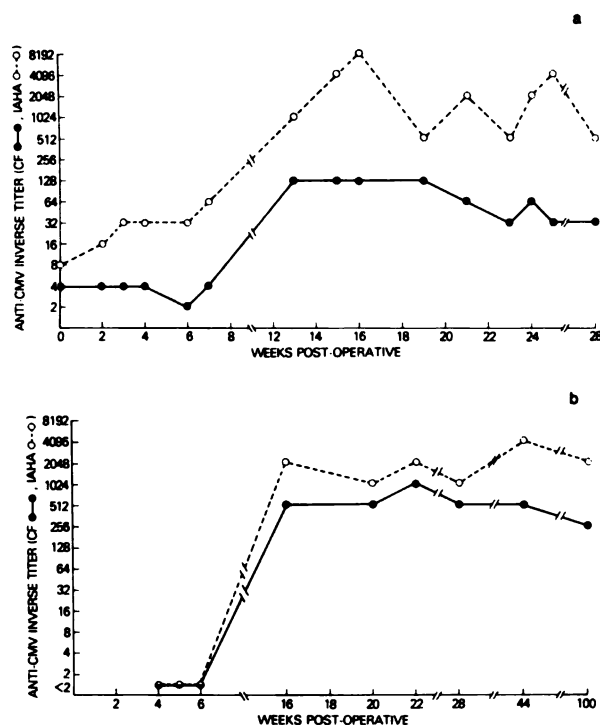


FIG. 3. Serial determinations of antibody to cytomegalovirus (anti-CMV) by complement fixation (CF) and immune adherence hemagglutination (IAHA) after transfusion in the sera of (a) a patient with pretransfusion anti-CMV and (b) a patient with no pretransfusion anti-CMV.

the preparation of a multivalent CMV antigen would make this test even more valuable.

In addition to enhanced sensitivity, IAHA required one-quarter of the CMV antigen reagent required for CF. Furthermore, since similar IAHA tests are available to detect antibody to hepatitis B surface (9) and core (13) antigens and hepatitis A antigen (11, 12, 14), theoretically, replicate dilutions of a single heat-inactivated serum sample could be tested for antibody to hepatitis and CMV antigens with the same technique in one microtiter plate. Since these viruses may be transmitted by transfusion, determination of these antibody responses is important to determine the etiology of transfusion-associated hepatitis and other syndromes. Perhaps, too, an IAHA test for antibody to Epstein-Barr virus, another agent transmitted by transfusion, can be developed, making serologic testing of post-transfusion patients even more efficient.

The only IAHA reagents difficult to ob-

tain, the indicator red blood cells, can be found by screening cells from many blood donors with blood type O. Once suitable donors are identified, their erythrocytes may be stored in four parts of Alsever's solution (vol/vol) for up to 1 month for use in IAHA. Unlike previously reported IAHA assays (9), no prozone phenomenon was observed in the application of IAHA to CMV antigen-antibody detection.

Finally, no major difference was noted in the kinetics of anti-CMV development between CF and IAHA in the two patients studied. In contrast to the earlier development of CF antibody than IAHA antibody to hepatitis A antigen (11, 12), significant rises in anti-CMV occurred at the same time, when measured by CF and IAHA.

Summary. The principle of complement-mediated immune adherence was applied to development of an immune adherence hemagglutination (IAHA) assay for antibody to cytomegalovirus (anti-CMV). Compared to complement fixation (CF), IAHA was

two to eight times more sensitive for the detection of anti-CMV. In paired serum samples from multiply transfused patients, the concordance between rises in anti-CMV titers detected by CF and IAHA was 0.671 ($P < 0.001$). Convalescent titers measured by IAHA were significantly higher than those determined by CF in serum pairs in which anti-CMV rises were demonstrated by both techniques, and IAHA more commonly revealed anti-CMV in pretransfusion serum samples. No difference was noted between the kinetics of CF and IAHA antibody development after transfusion. Immune adherence hemagglutination, we conclude, is an efficient, sensitive alternative to CF for the detection of anti-CMV.

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Curie	Ci	molar (concentration)	<i>M</i>
degree Celsius (Centigrade)	-°	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	dia	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	<i>N</i>
inch	in.	osmole	Osm
inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intrapertoneal	ip	parts per million	ppm
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